The role of regulatory T cells in early life immunity to BCG: influence of exposure to environmental mycobacteria

Thesis

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The Role of Regulatory T cells in Early Life Immunity to BCG: Influence of Exposure to Environmental Mycobacteria

Sarah Burl B.Sc M.Sc

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AFFILIATED RESEARCH CENTRE: MRC The Gambia, PO Box 273, Atlantic Road, Fajara, The Gambia, West Africa

COLLABORATING ESTABLISHMENT: The Jenner Institute, Old Road Campus Research Building, Oxford University-Nuffield Dept of Clinical Medicine, Oxford, OX3 7DQ, UK

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

ABSTRACT

BCG is the only licensed vaccine against tuberculosis (TB), yet its protective efficacy is variable. Exposure to non-tuberculous mycobacteria (NTM) has been shown to render BCG less effective. It was therefore hypothesised that exposure to NTM in early life may attenuate the immunogenicity of BCG, and that regulatory T cells (Tregs) may contribute to this reduced response.

Neonates were recruited at birth (n = 103) and randomised to receive BCG vaccine at birth or at 4½ months of age. Blood was collected at birth, 4½- and 9- months of age and immune responses were assessed by flow cytometry and multiplex bead array.

Exposure to NTM prior to BCG vaccination elicited Th1 and Th2 responses to PPD-tuberculin at 4½ months, including IL-10 induction that was not observed in those vaccinated at birth. Higher IFNγ production 4½ months post vaccination was observed in children vaccinated at birth compared to 4½ months of age. The results support the starting hypothesis and suggest that while $FOXP3^+$ Tregs are not involved in the attenuated response, NTM induced IL-10-producing Tregs may be responsible for the reduced IFNγ reactivity. Interestingly IL-13 was induced by BCG vaccination at both time points and was sustained until 9 months of age in those vaccinated at birth.

IFNγ and IL-10 both correlated with tuberculin skin test (TST) reactivity. The data suggests that IFNγ may initiate the response, but IL-10 controls the induration. It was also clear that higher 'cut offs' for the TST are more appropriate for defining possible *M.tb* infection in BCG vaccinated children in this environment.

Overall the results suggest that exposure to NTM attenuated the pro-inflammatory response when vaccination was delayed. Vaccination at birth induced a mixed Th1/Th2 response at 4½ months, but only the Th2 response was sustained at 9 months. Furthermore, IL-10 appears to play a critical role in regulating mycobacterial immunity.

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Dedicated to Prof. John Clark,

may he rest in peace

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ALRTI	acute lower respiratory tractinfection
APC	antigen presenting cells
APC	allophycocyanin
APL	alternative peptide ligands
aTregs	adaptive regulatory T cells
BCG	Bacillus Calmette Guerin
BrdU	bromodeoxyuridine
cAMP	cyclic adenosine monophosphate
CBA	cytometric bead array
CD	cluster of differeniation
CFP-10	culture filtrate protein-10kD
CFSC	carboxyfluorescin diacetate succinimidyl ester
CFU	colony forming units
CHS	contact hypersensitivity
CLA	cutaneous lymphocyte-associated antigen
CMV	cytomegalovirus
CRF	case report formd
CSIF	cytokine synthesis inhibitory factor
CTL	cytotoxic T lymphocytes
DC	dendritic cell
DP	CD4CD8 double positive T cells
DTH	delaed type hypersensitivity
DTwP	diphtheria, tetanus with pertussis combined vaccine
E	ethambutol
EAE	experimental autoimmune encephalitis
EBV	Epstein Barr virus
ELISA	enzyme-linked immunosorbent assay
ELISpot	enzyme-linked immunospot assay
EM	environmental mycobcateria
EPI	Extended Programme of Immunisation
ESAT-6	early secreted antigenic target 6-kDa protein
FITC	fluorescein isothiocyanate
FOXP3	forkhead winged-helix transcription factor
GCP	Good clinical practice
GDP	Gross domestic product
H	isoniozid
H_2O_2	hydrogen peroxide
Hb	haemoglobin
HBHA	heparin-binding hemagglutinin antigen
HBV	hepatitis B virus
HCV	hepatitis C virus
Hib	Haemophilis influenza B
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HSV	herpes simplex virus

ICH	Internation conference for harmonisation
ICS	intracellular staining
IFNγ	interferon gamma
IGF-1	insulin-like growth factor 1
IGRA	interferon gamma release assay
IL	interleukin
iNOS	inducible nitric oxide synthase
IPEX	immune dysfunction polyendocrinopthay enteropathy X linked
IWC	Infant Welfare Card
KO	knockout
LCMV	lymphocytic choriomeningitis virus
LGG	Lactobacillus rhamnosus GG
LIF	leucocyte inhibitory factor
LPS	bacterial lipopolysaccharide
LTBI	latent tuberculosis infection
M.tb	*Mycobaterium tuberculosis*
MAIS	*M. avium-intracellulare-scrofulaceaum*
MAP	M. avium subspecies paratuberculosis
MBP	myelin basic protein
MCH	mean cell haemoglobin
MCHC	mean cell haemoglobin concentration
MCV	mean cell volume
MDR-TB	multi-drug resistant TB
MHC	major histocompatibility complex
MRC	Medical Research Council
MS	multiple sclerosis
MV	measles vaccine
NK	natural killer cells
NKT	natural killer T cells
NMR	neonatal mortality rate
NO	nitric oxide
NOS2	nitric oxide synthase
NRBC	nucleated-red blood cell precursors
NSE	non-specific effects
nTegs	naturally occurring regulatory T cells
NTM	non-tuberculosis mycobacteria
OPV	oral polio virus
OVA	ovalbumin
PAF	platelet activating factor
PAMP	pathogen associated molecular patterns
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered serum
PCV	packed cell volume
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
pf	Plasmodium falciparum
PfTRAP	Plasmodium falciparum thrombospondin-related adhesive

	protein
PHA	phytohaemagglutinin
PPD	purified protein derivative
PPD-T	purified protein derivative tuberculin
PPP	per capita
PRR	pattern recognition receptors
R	rifampicin
RA	retinoic acid
RBC	red blood cell
RD1	region of difference 1
RNI	reactive nitrogen intermediate
ROI	reactive oxygen intermediate
RTE	recent thymic emigrants
SCC	Scientific Co-ordinating Committee
SEB	staphylococcal enterotoxin B
SEM	standard error of the means
SI	stimulation index
SSI	Statens Serum Institute
STAT-1	signal transducer and activator of transcription 1
T.U.	tuberculin units
TB	tuberculosis
TBQ	TB Questionnaire
TCR	T cell receptor
TGFβ	transforming growth factor beta
Th	T helper cell
TLR	toll-like receptors
TNFα	tumour necrosis factor alpha
TRAIL	TNF-related apoptosis-inducing ligand
TRECs	T cell receptor excision circles
Treg	regulatory T cells
TST	tuberculin skin test
UCB	umbilical cord blood
UNICEF	United Nations (International) Children's Fund
VIT	venom-specific immunotherapy
WBC	white blood cells
WHO	World Health Organisation
WT	wild type
XDR-TB	extensively drug resistant tuberculosis
YF	yellow fever
Z	pyrazinamide

CHAPTER 1

Introduction

1.1 MYCOBACTERIUM TUBERCULOSIS

1.1.2 Epidemiology of tuberculosis

Despite the introduction of vaccination against tuberculosis (TB) with a laboratory attenuated strain of *M.bovis* (Bacillus Calmette-Guérin vaccination (BCG)) in the 1920's, transmission of *Mycobacterium tuberculosis (M.tb)*, the causative organism of the disease has not yet been controlled. It is estimated that up to one third (2.2 billion) of the world's population may be infected with *M.tb*, (Sudre, ten Dam et al. 1992) and approximately 10% of these cases will progress to clinical disease during their lifetime, resulting in an estimated annual death rate of 1.8 million worldwide. With the emergence of HIV infections and drug resistant *M.tb*, these figures are likely to increase.

1.1.3 Microbiology of mycobacteria

M.tb is part of the genus *Mycobacterium* which are rod-shaped aerobic and non-motile bacteria (approximately 1 – 10 μm long) that are characteristically acid-alcohol fast, due to the high content of lipids comprised mainly of mycolic acid. Although considered Gram-positive bacteria with a peptidoglycan cell wall and lack of outer cell wall, Gram staining is negative due to the unique 'waxy' cell wall present in mycobacteria, and therefore this method cannot be used in diagnosis. The classical stain for mycobacteria is the Ziehl Neelsen stain with which mycobacteria stain bright red/pink (Shoub 1923). A natural division occurs between the slow growers (> 7 days per cell division) including *M.tb, M. avium, M. bovis, M. marinum, M. africanum, M. kansasii,* and *M. intracellulare,* and rapid growers (< 7 days per cell division) such as *M. smegmatis* and *M. fortuitum*. The pathogenicity of the various mycobacterial infections depends upon virulence factors that mediate survival inside the host. Those mycobacteria that cause tuberculosis form the *M.tb* complex and include *M.tb*, *M. bovis, M. africanum* and *M. microti*. Comparative genomics of the mycobacteria within the *M.tb* complex show that overlapping portions of the region of difference 1 (RD1) are absent from the *M. bovis* (BCG) attenuated strain (including

coding regions for early secretory antigen-6 (ESAT-6) and culture filtrate protein-10 (CFP-10)) suggesting that this region codes for important virulence factors (Mahairas, Sabo et al. 1996; Behr and Small 1999; Behr, Wilson et al. 1999).

Non-tuberculous mycobacteria (NTM) cause neither TB nor leprosy, but they may cause pulmonary or disseminated disease similar to TB, particularly in the immunocompromised host (e.g. *M. avium* and *M. marinum*). In most parts of the world TB is predominantly caused by *M.tb*, but in West Africa *M. africanum* causes almost half of the cases of pulmonary tuberculosis (Niobe-Eyangoh, Kuaban et al. 2003) and appears to have reduced virulence and T cell immunogenicity compared to *M.tb* (Mostowy, Onipede et al. 2004; de Jong, Hill et al. 2006). Recent studies have found 38% of all TB cases in The Gambia to be attributed to *M. africanum*, 63% of these were HIV-infected (de Jong, Hill et al. 2005). This preponderance of *M. africanum* associated TB disease in immunocompromised individuals suggests that *M. africanum* may be more like an opportunistic infection than *M.tb,* although disease severity is comparable (de Jong, Hill et al. 2005; de Jong, Hill et al. 2007).

1.1.4 Clinical pathology of tuberculosis

Tuberculosis is an air-borne infection which is commonly transmitted through coughing, sneezing or spitting aerosol droplets containing the organism. Exposure in 10 - 30% of contacts will lead to infection (Zhang 2008), and of those, 90% will become latently infected (LTBI) with the *M.tb* sitting dormant within macrophages (1995). The contained *M.tb* induce granuloma formation; a dynamic structure containing activated lymphocytes and continuously stimulated macrophages due to local tumour necrosis factor alpha (TNFα) production (Mohan, Scanga et al. 2001). Re-activation of *M.tb* occurs in approximately 10% of such latently infected individuals, often due to reduced immunocompetence from factors that include malnutrition, physical and mental stress, aging and immunosuppressive conditions (e.g. HIV) or therapies. Such individuals may

progress quickly to symptomatic disease, with signs including persistent cough, weight loss and night sweats. The recommended standard treatment for *M.tb* is 2 months with rifampicin (R), isoniazid (H), pyrazinamide (Z) and ethambutol (E), followed by 4 months with RH alone. Unfortunately resistance to anti-tuberculosis drugs has become a major public health issue in recent years (CDC 2008). Multi-drug resistant TB (MDR-TB) is defined as resistance to R and H and extensively drug-resistant TB (XDR-TB) is resistance to 3 or more classes of second-line drugs (CDC 2008).

1.2 IMMUNE RESPONSE TO MYCOBACTERIA

1.2.1 Innate response

The immune response to mycobacteria involves both the innate and the adaptive arms of the immune system. Upon inhalation of *M.tb* the bacteria are phagocytosed, predominantly by alveolar macrophages, facilitated by a number of molecules including cholesterol (Gatfield and Pieters 2000) and mannose receptors that recognise the mannose-rich cell wall of the mycobacteria (Bonar, Chmiela et al. 2005). Interactions between the pathogen associated molecular patterns (PAMPs) found on the organism with pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) on immune cells, initiate the innate immune response. Purified mycobacterial antigens preferentially interact with TLR2, possibly in combination with other TLRs. However, infection with whole bacilli evokes a more complex activation pattern involving at least TLR2 and TLR4 (Quesniaux, Fremond et al. 2004), although TLR1, 6, 8 and 9 have also been shown to be involved in *M.tb* recognition (Bulut, Faure et al. 2001; Bafica, Scanga et al. 2005; Davila, Hibberd et al. 2008). Control of infection is mediated by release of cytokines and chemokines that enhance recruitment of more leukocytes and activate phagocytosis of the mycobacteria, leading to engulfment within a phagosome. This phagosome fuses with a lysosome in the cell to form a phagolysosome containing microbicidal material including proteolytic enzymes, lysozyme and nitric acid. This combination of factors causes mycobacteria to be

broken down, within the phagocytes and antigenic fragments of the pathogen are loaded onto the Major Histocompatibility Complex (MHC) class II molecules and presented to T cells at the surface of the macrophage. Infected macrophages and dendritic cells (DCs) secrete various cytokines including IL-12 which initiate the Th1 immune response leading to increased IFNγ production. Early and rapid production of IFNγ from natural killer (NK) cells can initiate activation of macrophages. IFNγ activated macrophages are thought to control the infection at the localised site, whilst phagocytic dendritic cells migrate to the lymph node in an IL-12p40 dependent manner (Cooper and Khader 2008) and present antigen to cells of the adaptive immune system, polarising naïve T cells towards a Th1 response. In addition IFNγ activated macrophages trigger TNFα production, increased antigen presentation, increased production of reactive nitrogen intermediates (RNI) such as nitric oxide (NO) and reactive-oxygen intermediates (ROI), all leading to effective killing of mycobacteria (Chan, Chan et al. 2001). NO is produced by macrophages in response to IFNγ signalling from inducible nitric oxide synthase (iNOS) and has anti-mycobacterial properties in mice (Chan, Chan et al. 2001) but this is more controversial in humans. However, functional iNOS has been identified in the bronchoalveolar lavage of pulmonary TB patients (Nicholson, Bonecini-Almeida Mda et al. 1996), as has NO production from the exhaled air (Wang, Liu et al. 1998; Idh, Westman et al. 2008), suggesting that NO also plays a role in human disease.

IL-6 is also produced by macrophages and contributes to early *M.tb* immunity by promoting inflammation (Saunders, Frank et al. 2000) and Th17 T cell development in combination with transforming growth factor beta (TGFβ) (Djoba Siawaya, Beyers et al. 2009) (Awasthi and Kuchroo 2009). However IL-6 can also play an anti-inflammatory role by reducing the IFNγ response from uninfected macrophages in mice (Nagabhushanam, Solache et al. 2003). The locally activated macrophages also produce the immunosuppressive cytokine IL-10, which contributes to reduced IL-12 production (Hickman, Chan et al. 2002). Thus, it is most likely that the balance between the virulence

of the pathogen and the quality and magnitude of the host response that determines disease susceptibility or resistance.

1.2.2 Adaptive response

Presentation on MHC class II molecules induces a $CD4^+$ T cell response and polarisation towards a Th1, IFNγ response as mentioned earlier. Enhanced microbicidal activity of infected macrophages is predominantly mediated by this IFNγ from $CD4^+$ T cells (Flynn 2004). The role for $CD4^+$ T cells in protective immunity to TB is highlighted by the susceptibility of CD4-gene depleted mice and HIV-infected individuals (Corbett and De Cock 1996; Caruso, Serbina et al. 1999; Flynn 2004). In addition $CD4^+$ T cells appear to have *M.tb*-specific cytotoxic activity where restriction of *M.tb* growth is granule dependent suggesting CTL activity of CD4+ T cells may play a role in immunity to TB (Canaday, Wilkinson et al. 2001).

Other T cell populations can also be involved in the protective response including $CD8^+$, CD1-restricted T cells (invariant NKT cells) and gamma-delta (γδ) T cells, the mechanisms of which are less clear (Kaufmann, Baumann et al. 2006).

An increasing number of studies have demonstrated the importance of $CD8^+$ T cells in mycobacterial immunity through the production of protective cytokines such as IFNγ and TNFα (Smith and Dockrell 2000; Kaufmann 2001; Kaufmann and McMichael 2005). CD8 T cells specific for epitopes in ESAT-6 and with cytolytic activity have been found in TB patients (Lalvani, Brookes et al. 1998). These CD8 T cells have also been found to localise to sites of infection (Caccamo, Meraviglia et al. 2006). Interestingly, studies in The Gambia have shown reduced perforin production from $CD8^+$ and reduced cytotoxic T cell activity in TB patients (Smith, Klein et al. 2000) along with reduced IFNγ and TNFα. The presence of IL-4 producing $CD8^+$ T cells was only evident in the TB patients suggesting alteration in the Th1 and Th2 cytokine balance in $CD8^+$ T cells during TB disease (Smith, Klein et al. 2002). Although *M.tb* primarily resides in vacuoles, it has been

proposed that antigens from *M.tb* reach the cytosol by escape of whole organism (McDonough, Kress et al. 1993), regurgitation of processed antigen (Pfeifer, Wick et al. 1993), and/or movement of soluble proteins through pores within the membrane of TB phagosome (Mazzaccaro, Gedde et al. 1996; Cho, Mehra et al. 2000) which may explain the contribution of CD8 T cells in *M.tb* infection.

1.2.3 Cytokine responses

During *M.tb* infection and disease progression, signs of both activated and suppressive cytokine production are present highlighting the complexity of immunity to TB.

Th1 cytokines

Human studies have shown that individuals with mutations in the genes that code for IFNγ or the IFNγ receptor (IFNGR) exhibit increased susceptibility to *M.tb* infection (Casanova, J *et al*, 2002)(Newport, Huxley et al. 1996) indicating the importance of this cytokine in anti-mycobacterial immunity.

However evidence in animals, and more recently in humans, suggest this may not be the case. Revaccination with BCG at 6 and 9 weeks in cattle induced increased IFNγ and IL-2 cytokine responses to PPD, but these were associated with greater lung and lymph node tuberculosis lesions than those vaccinated once at birth although still less than the unvaccinated group (Buddle, Wedlock et al. 2003). Similarly a lack of correlation between IFNγ responses to PPD and protection against tuberculosis was found in the non-human primate model (Langermans, Andersen et al. 2001). However mice studies have shown that limiting IFNγ, or the repertoire of the IFNγ-producing T cells, allows control of short term infection, but subsequent maintenance of control is lost suggesting that different protective mechanisms may be involved at different stages of infection (discussed in (Cooper and Khader 2008)). Recent studies have illustrated that many other cytokines have

immunoregulatory roles in the control of infection (Dockrell 2007; Goldsack and Kirman 2007). Goldsack *et al* presents evidence that memory CD4 T cells can protect against tuberculosis in the absence of IFNγ and suggest IL-10 and regulatory T cells may be involved (Goldsack and Kirman 2007).

Th17 cytokine

IL-17 is a pro-inflammatory cytokine that is predominantly produced by Th17 differentiated $CD4^+$ T cells that develop in the presence of IL-23, although γδ T cells also produce IL-17 during mycobacterial infection (Lockhart, Green et al. 2006; Khader, Bell et al. 2007; Khader and Cooper 2008). TGFβ together with IL-6 or IL-21 are required to initiate Th17 cells suggesting a link between the innate response and regulatory T cells (Awasthi and Kuchroo 2009). IL-17 is increased in IFNγ deficient mice following infection with BCG, but these mice are unable to control infection suggesting that IL-17 is not required for protection (Cruz, Khader et al. 2006). In support of this, production of IL-17 was observed as a primary response to a known non-protective vaccine in mice, compared to a lack of IL-17 production to a protective vaccine (Romano, D'Souza et al. 2006). In contrast, IL-17 has been shown to have a role in the secondary response against TB in mice. Protection from a peptide-based vaccine was found to be dependent on IL-17 production as part of a memory response, alongside a reduction in IFNγ (Khader, Bell et al. 2007). Recently human studies have observed a reduction of IL-17 producing $CD4^+$ T cells in the peripheral blood of TB patients compared to TB contacts further supporting the idea that IL-17 may play a role in protection against TB (Scriba, Kalsdorf et al. 2008; Sutherland, Adetifa et al. 2009). The concept of Th1 and Th17 T cells both being induced by *M.tb* infection in response to IL-12p40 and IL-12p70 is discussed in the review by Cooper and Khader (Cooper and Khader 2008). The conflicting studies suggest that the timing of IL-17 and IFNγ production e.g. as a primary or a memory response may be crucial to the type of immune response triggered and consequently the protective effect

against TB.

Th2 cytokines

Th2 cytokines can play a negative role in *M.tb* infection (Seah, Scott et al. 2000). IL-4 can inhibit IFNγ production in mice (Heinzel, Sadick et al. 1989) (Buccheri, Reljic et al. 2007). Indeed, IL-$4^{-/-}$ KO mice had substantially reduced infection in lungs and spleen for up to 8 weeks (Buccheri, Reljic et al. 2007). However, TB patients often exhibit both a Th1 and a Th2 response to *M.tb*, which is not inconsistent with the classic Th1/Th2 paradigm (Jung, Ryan et al. 2003) (Kidd 2003). IL-13 shares a receptor subunit with IL-4 (IL-4Rα) and therefore the function of these cytokines is often linked. Indeed IL-13 concentrations are increased in TB patients (Seah, Scott et al. 2000), and high levels are associated with a rapid response to TB treatment, suggesting a protective effect of IL-13 (Djoba Siawaya, Beyers et al. 2009). The mechanism of action is not clear, but it may assist in preventing autophagic killing of *M.tb* in macrophages (Harris, De Haro et al. 2007).

Suppressive cytokines

One of the strongest inhibitors of IFNγ is IL-10. In humans, elevated levels of IL-10 were observed in the serum of TB patients compared to contacts, and it was suggested that IL-10 may play a role in re-activation of TB (Verbon, Juffermans et al. 1999). IL-10 is produced from many cell types of both the innate and adaptive immune system, making it difficult to interpret the role of IL-10 in *M.tb* infection and disease. Increased IL-10 from macrophages is associated with an increased tendency to develop TB (Awomoyi, Marchant et al. 2002). Interestingly, T cell clones from bronchoalveolar lavage fluid that expressed both IFNγ and IL-10 have been observed, which might be important in balancing the immune response (Gerosa, Nisii et al. 1999). Interestingly human IL-10 was first cloned from an IFNγ secreting T cell clone as cytokine synthesis inhibitory factor (CSIF) (Vieira,

de Waal-Malefyt et al. 1991). Several studies have observed these Th1 IL-10 secreting cells, and it has been suggested that IL-10 secreting antigen specific T cells may be activated later during infection after the initial pro-inflammatory IFNγ response from the same cells (reviewed in (O'Garra and Vieira 2007)). *M.tb* can also induce production of the regulatory cytokine TGFβ from macrophages, which in turn suppresses Th1 responses during infection (Toossi, Young et al. 1995; Guyot-Revol, Innes et al. 2006). This may explain why IFNγ production in PBMC cultures from TB patients can be enhanced by TGFβ inhibition (Hirsch, Ellner et al. 1997). TGFβ induced *in vitro* following PPD stimulation can induce IL-10 production from monocytes and potentiate the downregulation of IFNγ (Othieno, Hirsch et al. 1999).

Finally further studies have identified other cytokines that may play a role in the T cell mediated control of *M.tb* infection and disease including IL-27, IL-18 and IL-8 (Yamada, Shijubo et al. 2000) (Wu, Huang et al. 2007), (Sutherland, Adetifa et al. 2009), reviewed in (Cooper and Khader 2008). However, the exact signature of biomarkers and the time such factors act to provide protection or susceptibility to disease remains to be fully elucidated.

1.3 ANIMAL MODELS OF M.TB

Host-pathogen interactions during *M.tb* infection are difficult to study in a human system, mainly due to difficulties accessing the site of infection and the complexity of the disease process. The mouse is the most commonly used experimental animal model for many diseases including tuberculosis. The ability to access tissue sites of infection and the wide range of immunological reagents and transgenic animals available make the mouse ideal for mechanistic studies, although it is not necessarily the best model of *M.tb*. The mouse is not a natural host for *M.tb* and the course of infection is quite different to the human disease. Over the years a number of alternative animal models have been identified,

all with advantages and disadvantages. The first stages of infection are similar in most models, but latency, granuloma formation, caseation of the granuloma, cavity formation and TB disease patterns vary (Figure 1.1). *M.tb* infection in mice does not lead to latency and granuloma formation, the guinea pig forms granulomas but does not enter a state of latency, and the rabbit harbours infected macrophages in granulomas leading to cavity formation but lack reagents and are expensive to keep (Capuano, Croix et al. 2003). Cattle are the natural host for a mycobacterial infection similar to TB (bovine tuberculosis) which develops with many of the key features of human TB. Calves are immunocompetent at birth and naturally sensitised to environmental antigens, which makes this model particularly useful for comparative human infant studies in geographical locations with high levels of NTM, however these models are expensive and the availability of reagents is limited (Buddle, Skinner et al. 2005). The zebrafish has provided a model that can be infected with *M. marinum* (a pathogen of ectoderms closely related to *M.tb*) leading to a similar course of infection and disease to human TB. Fish have a complex adaptive immune system akin to that of mammals and their transparency for the first three weeks of life means that it is possible to trace the migration of cells during development (Pozos and Ramakrishnan 2004). The non-human primate model of tuberculosis has also been used for many years, but the cost and biocontainment required have limited their use. Cynomolgus monkeys can be reproducibly infected with low doses of *M.tb* via a flexible bronchoscope leading to a disease very similar to that seen in humans including latent infection, granuloma and cavity formation (Capuano, Croix et al. 2003). With the advent of genomics and increasing bioinformatics expertise, mathematical models of infection and disease have shown promise in furthering our understanding of the mechanisms of granuloma formation and TB disease (Morel, Ta'asan et al. 2006; Ray, Flynn et al. 2009). This approach could help to reduce the need for animal experimentation, and promote a more targeted approach to studying tuberculosis.

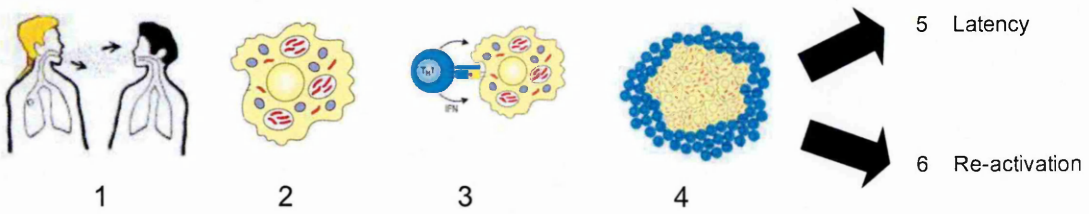


Figure 1.1: Infection process of Mycobacterium tuberculosis (M.tb). *M.tb infection is initiated by transmission of M.tb infected droplets by aerosol (1), 7 – 10 days later M.tb starts multiplying in macrophages (2), the lymphocytes are activated which activate macrophages and convert a tuberculin skin test to positive (3). Granulomas form that have a centre of low pH and low oxygen which inhibits M.tb growth (4) and contains the M.tb in a state of latency (5). The centre of the granuloma can become necrotic and form the cheesy consistency described as caseation. Upon re-activation, M.tb multiplies in the centre of the granuloma and leaks into the extracellular space, further multiplying and leading to cavity formation and dissemination of disease (6). Diagrams obtained from (Janeway 2005).*

1.4 IMMUNE EVASION BY M.TB

M.tb has co-evolved with its human host for thousands of years with the oldest human remains showing signs of *M.tb* infection being 9,000 years old (Hershkovitz, Donoghue et al. 2008). This has allowed ample time for *M.tb* to develop mechanisms to evade the immune response of the host (reviewed in (Flynn and Chan 2003)). These mechanisms are illustrated in Figure 1.2. As a pathogen that resides inside macrophages, the most obvious strategy has been to avoid destruction within the cell once it has entered, thereby allowing latency to be established. Murine studies have shown the importance of the nitric oxide synthase 2 (NOS2)-dependent pathway generating toxic RNIs as a method of controlling pathogens within macrophages. In iNOS $^{-/-}$ KO mice infection with *M.tb* is

associated with a significantly higher risk of dissemination and mortality (reviewed in (Chan, Chan et al. 2001)). Removal of these RNIs using nitric oxide synthase inhibitor aminoguanidine after infection with a virulent strain of *M.tb* resulted in susceptibility and reactivation of TB (Flynn, Scanga et al. 1998). Evasion of RNI toxicity has been shown in murine models to occur by the production of antioxidant products. In humans, it has been difficult to induce NO in many *in vitro* systems making it difficult to confirm the murine work. However, the presence of iNOS has been found *in vivo* in the human lung (Nathan 2002) and alveolar macrophages (Nicholson, Bonecini-Almeida Mda et al. 1996; Wang, Liu et al. 1998) but it is unclear what RNI evasion strategies *M.tb* applies in humans. Both maturation of, and the environment within, the phagolysosome are altered by *M.tb,* affecting the phagosomal biogenesis and allowing the bacilli to avoid the normal antimicrobial effects (Russell 2001). Furthermore, IFNγ induced MHC class II upregulation and thus presentation of antigen to the surface of macrophages is also inhibited by *M.tb* infection (Hmama, Gabathuler et al. 1998). This inhibition is controlled by a 19kD fraction of *M.tb* that acts via TLR2 pathway within the phagosome (Noss, Pai et al. 2001). However this 19kD protein can also induce Th1 cytokine production associated with a good immune response. It is possible that these opposing effects occur at different stages of infection and that the inhibitory effect dominates during the chronic stages (Flynn and Chan 2003).

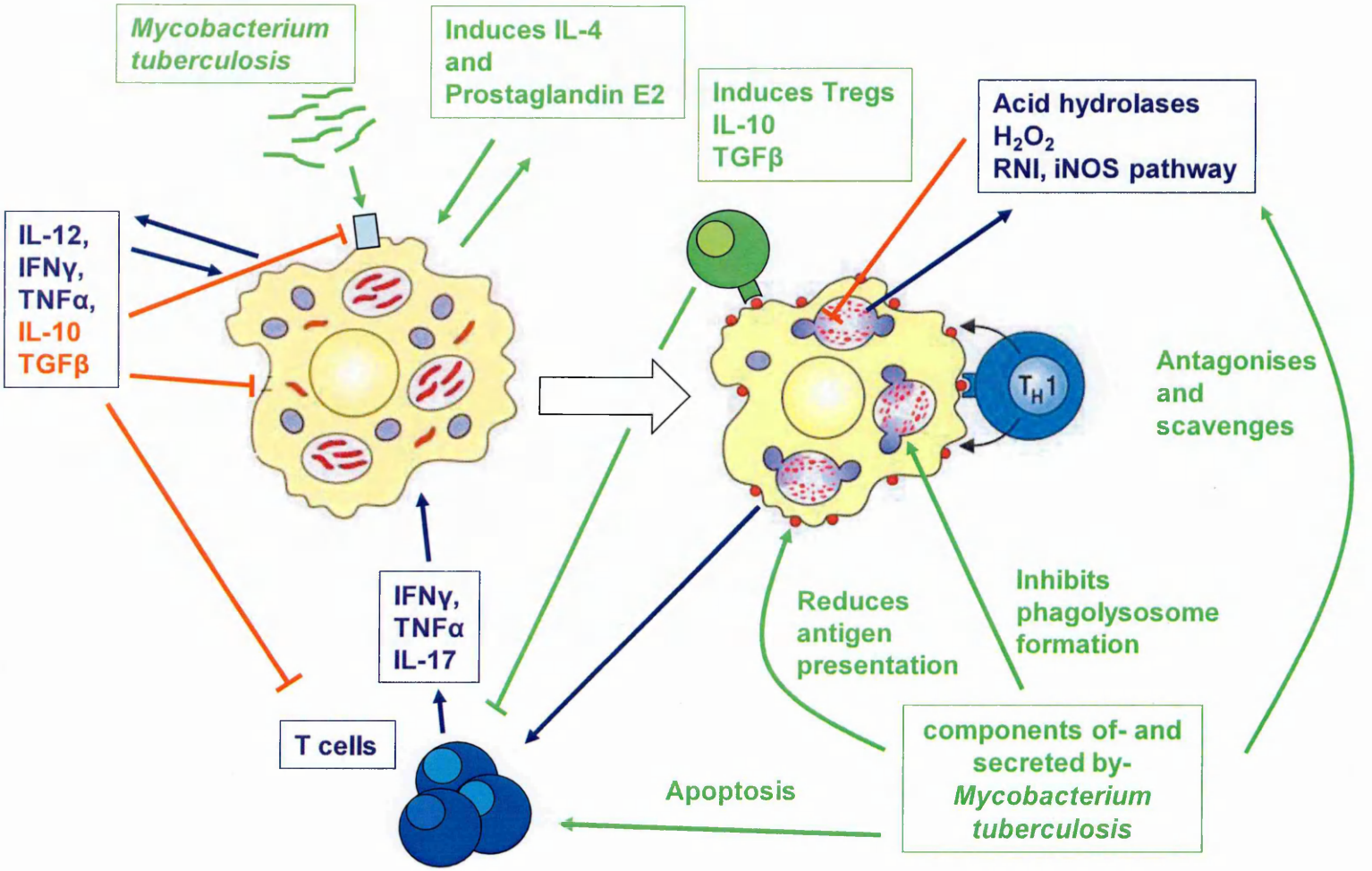

Mycobacterium tuberculosis
Induces IL-4 and Prostaglandin E2
Induces Tregs IL-10 TGFβ
Acid hydrolases
H_2O_2
RNI, iNOS pathway
IL-12, IFNγ, TNFα, IL-10 TGFβ
T_H1
Antagonises and scavenges
IFNγ, TNFα IL-17
Reduces antigen presentation
Inhibits phagolysosome formation
T cells
Apoptosis
components of- and secreted by- Mycobacterium tuberculosis

Figure 1.2: A schematic summary of mycobacterial infection of macrophages. *Macrophages phagocytose mycobacteria inducing an innate response that includes TNFα, IL-12, IL-6 and IFNγ production and recruitment of other immune cells to the site of infection. Mycobacteria are taken up into phagosomes that fuse with lysosomes forming a phagolysosome. The resulting M.tb epitopes are transported to the surface and loaded onto MHC class II molecules where they can initiate T cell activation. T cells then proliferate, produce cytokines and activate further macrophages. Induction of suppressor cytokines act to balance the immune response and limit immunopathology. Mycobacterium tuberculosis (M.tb) uses various mechanisms to evade the pro-inflammatory immune response as illustrated in green. Blue arrows = activation, red arrows = inhibition, green arrows = M.tb evasion strategies. (Macrophage diagrams (Janeway 2005)).*

1.5 PREVENTION OF TUBERCULOSIS

1.5.1 Bacillus Calmette Guérin (BCG) vaccine

BCG was first given as an oral vaccine for humans in 1921 to protect against tuberculosis. BCG consists of a live attenuated strain of *Mycobacterium bovis* that has been passaged a number of times over many years, but were originally maintained in Paris, France. The continual growth of these cultures has introduced various undefined genetic mutations and changed the activity and virulence of the vaccine to establish an attenuated form (reviewed in (Chen 2005)). In 1929, a tragic accident in Lübeck, Germany occurred when a virulent strain of TB was introduced into the vaccine and killed 29% of the 252 children vaccinated (reviewed in (Chen 2005)). From this time onwards the oral vaccine was withdrawn from the market and replaced with the intradermal vaccination route still used today (Andersen and Doherty 2005). This method of administering BCG produced greater reliability and reproducibility than the oral vaccine although there may be differences in the responses. Oral vaccination induces both cellular and humoral mucosal responses (Monteiro-Maia, Ortigao-de-Sampaio et al. 2006) whereas this may not be the

case with intradermal vaccination.

Distribution to several laboratories around the world has led to a number of different strains being maintained (reviewed in (Behr and Small 1999)) although today more than 90% of global BCG production is made up of either Russian BCG-1, Tokyo 172-1, Danish 1331, Moreau RDJ or Pasteur 1173-P2 sub strain 'seed lots' maintained in several laboratories in lyophilised batches (Ho 2004).

BCG is administered according to WHO guidelines which differ according to geographical location. Several developed, low risk countries e.g. US and most of Europe do not vaccinate individuals with BCG unless they are part of a high risk population within the country. However, WHO recommends that in countries with a high risk of TB, children should be vaccinated on first contact with a healthcare worker, most often at birth (WHO 2004).

Protective effect of BCG

The protective efficacy of BCG varies from 0-80%, which is highly associated with latitude (meta-analysis (Colditz, Berkey et al. 1995)). BCG efficacy against tuberculosis is poorer in vaccinated populations that live in rural areas closer to the equator than those further away or living in urban settings (Colditz, Berkey et al. 1995; Fine 1995). A high rate of protection (78%) was demonstrated in the UK amongst 14 to 15 year olds (Hart 1967), whereas no protection against TB was observed in Malawian studies (1996). Nevertheless, although long term protection in adulthood may be poor, BCG is thought to provide good protective efficacy against extrapulmonary severe forms of TB in childhood (Sudre, ten Dam et al. 1992). Moreover, TB patients that have been previously vaccinated with BCG elicited healed localised pulmonary lesions, whereas unvaccinated TB patients had more disseminated lesions (Sutherland and Lindgren 1979). This would suggest that BCG reduces the incidence of disease, but does not prevent infection.

The largest trial of BCG efficacy took place in Chingleput, India in the 1970s, and

showed very disappointing results (WHO 1979; 1980). The entire population of Chingleput of about 360,000 people were included in the trial. Two thirds were vaccinated with BCG (Danish and French) at two doses (standard 0.1 mg and low dose 0.01 mg) and the remaining third were not vaccinated. The incidence of infection was measured at baseline and at 2 follow up time points (6 months apart) and then at 2½ year intervals. Although it was predicted that BCG would elicit 80% protection, after the fifth 2½-year follow up interval, there were similar numbers of TB cases among both the vaccinated and unvaccinated groups, although the incidence for both groups was lower than expected (Hitze 1980; Tripathy 1983). Several possible theories have been developed to explain these variable results which are reviewed in (Smith, Wiegeshaus et al. 2000). One theory that was proposed was that the high level of non-tuberculous mycobacteria (NTM) present in the environment may have provided a degree of protection in the unvaccinated group similar to that of BCG. Skin tests to PPD-B revealed that 95% of the population included in the trial were reactors to NTM suggesting there was no real placebo (1980).

Reasons for variability of BCG protection

The reason for the variability of protection against TB across the world is still unclear but includes differences in the dose administered (Power, Wei et al. 1998), different strains of BCG (Lagranderie, Balazuc et al. 1996; Davids, Hanekom et al. 2006; Wu, Huang et al. 2007), the age at vaccination (Tripathy 1983) or methodological practices (Clemens, Chuong et al. 1983).

Effect of NTM on BCG efficacy

However, one of the most widely accepted hypotheses is that natural immunity to NTM in the environment renders BCG less effective or masks its protective effect (Comstock and Palmer 1966; Rook, Bahr et al. 1981; Stanford, Shield et al. 1981; Smith, Wiegeshaus et al. 2000).

A number of animal studies support this hypothesis: Mice exposed to *M. avium* complex prior to BCG vaccination showed inhibition of BCG bacterial growth in the lung and spleen, and inhibition of BCG specific immune responses by *ex vivo* IFNγ ELISpot assay (Brandt, Feino Cunha et al. 2002). This led to the proposed mechanism that prior sensitisation to mycobacterial antigens may lead to rapid elimination of BCG before it can generate a protective immune response. Several murine and cattle studies also showed that pre-sensitisation with *M. avium* was associated with poor vaccine responses to BCG (Buddle, Wards et al. 2002; Young, Slobbe et al. 2007). Oral infection of mice with *M. avium* prior to BCG parenteral immunisation decreased IFNγ secretion by splenic lymphocytes in response to overnight stimulation with PPD. This reduced IFNγ response was evident when BCG was given at 16 weeks of age although only statistically significant after 24 weeks of pre-sensitisation. Interestingly pre-sensitisation with *M. avium* 16 and 24 weeks prior to BCG vaccination increased IgG1 and IgG2a PPD-specific antibody responses compared to the PBS sensitised group suggesting cross-reactive mycobacteria may imprint an inappropriate, type 2 response on the immune system which negatively influences subsequent responses to BCG vaccination (Young, Slobbe et al. 2007).

In contrast *M. avium* can prime the immune response of calves to BCG vaccination. Twelve weeks after *M. avium* sensitisation, cattle were vaccinated with BCG. Fourteen weeks after vaccination increased IFNγ production in response to PPD-*bovis* was observed in the sensitised group and appeared more rapidly than the BCG alone group, however the PPD-*bovis* skin test reactivity was similar between the groups (Howard, Kwong et al. 2002). Similar findings in guinea pigs showed that BCG and MAI (*M. avium-intracellulare*) elicited similar levels of protection from low virulence strains of *M.tb* (Edwards, Goodrich et al. 1982). Interestingly, a study in cattle showed that 2/6 animals that had been pre-sensitised to *M. avium*, vaccinated with BCG 12 weeks later and then challenged with *M. bovis* at 24 weeks, had a positive tuberculin skin test (PPD-*avium* and *bovis*) at 36 weeks compared to 6/6 cattle that were not pre-sensitised but these animals

had fewer lesions and numbers of tissues containing viable *M.bovis* suggesting pre-sensitisation with mycobacterial antigens primed the immune response and enhanced BCG protection from *M.bovis* (Thom, Howard et al. 2008).

In addition earlier guinea pig studies showed that exposure to NTM of various types (*M. avium, M. kansasii and M. fortuitum*) prior to BCG vaccination had no effect on survival after *M.tb* challenge (Palmer and Long 1966).

Human studies in Malawi suggested that in adults a good IFNγ response to NTM was related to reduced BCG specific IFNγ responses, and therefore most likely less protection against tuberculosis (Black, Dockrell et al. 2001). Infant studies in The Gambia showed that 22% of non-BCG vaccinated, non-TB diseased infants exhibited a positive tuberculin skin test (TST) of > 5 mm induration at 3 months (Ota, Goetghebuer et al. 2006), suggesting priming by environmental NTM. Furthermore, good PPD-specific IFN-γ responses were observed *in vitro* in BCG naïve 2 month old infants, albeit at significantly lower levels than those immunised with BCG at birth (Vekemans, Ota et al. 2004).

Effect of age on BCG vaccination

As mentioned previously, in TB endemic areas WHO recommends that children should be vaccinated on first contact with a healthcare worker, most often at birth (2004). Despite the immaturity of the neonatal immune system, Weir *et al* have shown that vaccination at birth or during adolescence induces immunological memory to mycobacterial antigens 14 years after vaccination. However peripheral responses to PPD-tuberculin waned from 3 months to 12 months and from 12 months to 3 years post vaccination (Weir, Gorak-Stolinska et al. 2008). Strong Th1 responses to PPD-tuberculin were also observed in Gambian infants vaccinated at birth that were similar to those seen in adults (Marchant, Goetghebuer et al. 1999; Vekemans, Amedei et al. 2001), and these remained present at 1 year although at a lower level than 2 months after vaccination (Marchant, Goetghebuer et al. 1999). Further details in relation to vaccination in early life

will be discussed later.

BCG booster vaccination

Booster doses of BCG to augment the primary response have remained a contentious issue. A number of studies have failed to detect any protective effect of BCG revaccination (Sepulveda, Parcha et al. 1992; 1996; Chihota, Nyazema et al. 1998; Tala-Heikkila, Tuominen et al. 1998; Leung, Tam et al. 2001), although it has been shown to provide protection against leprosy (1996). It is assumed that the initial immune response prevents the booster vaccine to replicating in the host. However two studies from Eastern Europe conclude that revaccination with BCG may have some beneficial effects (Kubit, Czajka et al. 1983; Lugosi 1987). Interestingly, in cattle revaccination at 6 weeks following prior vaccination at birth reduced protection in calves, possibly by inducing pro-inflammatory immunopathology (Buddle, Wedlock et al. 2003). It may therefore be crucial to determine an appropriate time between vaccination and revaccination in order to optimise protection against TB. This may also be important when designing new vaccines using BCG as a vector (Kaufmann, Baumann et al. 2006).

1.5.2 Other vaccines for prevention of TB

The inconsistency of protection from BCG has led to further vaccines being developed that aim to replace the BCG or boost the BCG response. The leading TB vaccines are based on live mycobacterial vaccines largely by attenuating *M.tb* or modifying BCG, and subunit vaccines using a immunodominant viral vector or adjuvant to increase their immunogenicity (reviewed in (Andersen and Doherty 2005)).

One of the recombinant BCG vaccines expresses the listeriolysin O protein from *Listeria monocytogenes* (rBCGΔUre:CHly^{+}). The listeriolysin O protein damages the phagosomal membrane, allowing mycobacterial protein to be presented by MHC class I and permitting strong CD8+ T cells responses. This has exhibited better protection against virulent *M.tb* compared to BCG in the murine model (Conradt, Hess et al. 1999; Grode,

Seiler et al. 2005). In September 2008, human Phase I trials in adults commenced in Germany.

Many of the vaccines being developed contain Ag85, an immunogenic antigen from *M.tb*. MVA-Ag85 is a live, recombinant, replication-deficient vaccinia Ankara virus, expressing Ag85. As BCG confers protection against disseminated disease in childhood (< 10 years of life) removing it from novel vaccine strategies may not be appropriate. Therefore the BCG booster strategy has gained considerable interest in developing vaccines against TB. Phase 1 trials in mice of BCG prime followed by MVA-Ag85 boost resulted in stronger immunogenicity than BCG alone (McShane, Brookes et al. 2001). In addition, human Phase I studies found naturally acquired anti-mycobacterial immunity after vaccination with the same prime-boost strategy (McShane, Pathan et al. 2004). This vaccine is the most developed novel TB vaccine to date; it is currently in Phase IIa trials in a population of young infants in The Gambia (McShane, H and Ota, MO personal communication) and Phase IIb trials of this vaccine have recently commenced (March 2009) in South Africa with 2800 BCG vaccinated, 4 month old infants (McShane, H personal communication).

If BCG is to be used as a prime in many novel TB vaccine strategies it is important to understand its immunogenicity especially in a young population within a setting that is most likely to benefit from the new vaccine. As mentioned earlier, vaccination on a background of NTM exposure may influence vaccine efficacy. For this reason the testing of novel vaccines in a setting of high exposure to NTM is essential.

1.6 HYGIENE HYPOTHESIS

In a similar way that NTM may affect BCG vaccination it has been postulated that NTM may also play a role in the controversial 'hygiene hypothesis'. Originally formulated by Strachan *et al* in 1989, it was proposed that a lower incidence of infection in early childhood could explain the rise in allergic diseases in the developed world and the lower

incidence of allergy in developing countries with increased environmental microbial exposure (Strachan 1989). Rook *et al* proposed that exposure to mycobacteria with low pathogenicity, controlled by immunoregulatory mechanisms, may protect against later atopic disease (Rook, Hamelmann et al. 2007). This is supported by several studies showing an inverse relationship between atopic disorders and exposure to infectious pathogens in early life (Shaheen, Aaby et al. 1996; Matricardi 1997; Shirakawa, Enomoto et al. 1997; van den Biggelaar, van Ree et al. 2000). These studies include the effect of mycobacteria which can also be shown with BCG vaccination. Indeed, Aaby *et al* have shown that those with a BCG scar are less likely to have atopy than those without (Aaby, Shaheen et al. 2000). Defects in regulatory pathways in allergy and autoimmune diseases would support this idea. Suppression of allergen-induced eosinophilia in mice can be brought about by infection with *M.tb* (Erb, Holloway et al. 1998). In addition, vaccination with *M. vaccae* led to improved atopic dermatitis in children with moderate-to-severe disease (Arkwright and David 2001). The proposed mechanism behind this theory is based on the increased maturation of dendritic cells driving a suppressive Treg response (regulatory DCs), rather than a Th1 or Th2 inflammatory response (Rook, Hamelmann et al. 2007). The possible regulatory mechanisms involved in the hygiene hypothesis are dicussed later in this chapter.

1.7 REGULATION OF IMMUNE RESPONSES

1.7.1 Regulatory T cells (Tregs)

Distinguishing 'self' from 'non-self' is tightly controlled in the thymus by negative selection, however self reactive T cells can escape this process requiring a second control mechanism. Suppressor T cells were first postulated in the 1970s (Gershon, Cohen et al. 1972) as a mechanism to provide this tight regulation and prevent autoimmunity. Difficulties with identifying and characterising these cells led to the demise of this area of research (Moller 1988) and it wasn't until the mid-1990s that Tregs were identified as

distinct populations (Sakaguchi, Sakaguchi et al. 1995). Most of the initial studies examined the role of Tregs in autoimmunity, but later it became evident that Tregs were likely to be involved in all immune responses. A number of subsets of Treg populations are thought to exist, one of the most widely studied being those that possess the α-chain of the IL-2 receptor (CD25) that are designated naturally occurring Tregs (nTregs) since they are derived from the thymus (Sakaguchi, Fukuma et al. 1985; Sakaguchi, Sakaguchi et al. 1995). Adaptive or inducible Tregs (aTregs or iTregs) are peripherally induced in response to exogenous antigen, and are generally defined by their cytokine production (Groux, O'Garra et al. 1997).

Phenotype of Tregs

Mouse studies indicate that the entire subset of $CD4^+CD25^+$ T cells have regulatory properties, but human Tregs are thought to be confined to the $CD4^+CD25^{high}$ subset which consists of ~ 5% of the total $CD4^+$ T cell population, and frequently co-expresses other markers including intracellular CTLA-4, GITR, CD62L, neuropilin-1 and LAG-3 (Bruder, Probst-Kepper et al. 2004; Huang, Workman et al. 2004; Ochando, Yopp et al. 2005) (reviewed in (Wing, Fehervari et al. 2006)). To complicate matters CD25, and many of the Treg markers described to date are also markers of T cell activation and therefore not specific to Tregs. FOXP3, a forkhead/winged-helix transcription factor is expressed in this population and natural mutations in this gene have been shown to cause uncontrolled activation of T cells and dysregulation of immune function in both mice (termed 'Scurfy' (Brunkow, Jeffery et al. 2001)) and humans (immune dysfunction, polyendocrinopathy, enteropathy, X-linked or IPEX (Bennett, Christie et al. 2001)). Nomenclature for FOXP3 has been standardised to illustrate the human gene (*FOXP3*), the mouse gene (*Foxp3*) and protein expression from human and mouse (FOXP3) (Coffer and Burgering 2004). Expression of *Foxp3* in transgenic mice and ectopic expression in human cells has been shown to induce regulatory activity (Fontenot, Gavin et al. 2003; Hori, Nomura et al. 2003;

Fontenot, Rasmussen et al. 2005), and although the evidence suggests that expression of FOXP3 in any cell can confer suppressive activity, it has recently been shown to be upregulated upon activation (Gavin, Torgerson et al. 2006; Allan, Crome et al. 2007; Popmihajlov and Smith 2008) without inducing suppression (Morgan, van Bilsen et al. 2005; Allan, Crome et al. 2007). However this is a transient effect, peaking within 24 - 48 hours of polyclonal TCR stimulation *in vitro* (Gavin, Torgerson et al. 2006) (Morgan, van Bilsen et al. 2005), and returning to baseline levels by 5 - 10 days (Gavin, Torgerson et al. 2006; Popmihajlov and Smith 2008). FOXP3 expression has also been identified in human CD8 T cells (Roncador, Brown et al. 2005) (Wang, Ioan-Facsinay et al. 2007) which also show suppressive activity (Bisikirska, Colgan et al. 2005; Morgan, van Bilsen et al. 2005)(Cosmi, L *et al*, 2003) and within $CD4^+CD25^-$ T cells, possibly those committed to becoming Tregs (Morgan, van Bilsen et al. 2005). This evidence suggests that although FOXP3 is a good marker of suppressive Tregs, this marker cannot be relied upon to define Tregs. Combined gene expression microarray, flow cytometry and functional assays identified an inverse correlation between FOXP3 with the IL-7 receptor (CD127) (Liu, Putnam et al. 2006). It was shown that $CD4^+CD127^-$ T cells were as suppressive as the classic $CD4^+CD25^{high}$ population, and at present $CD4^+CD25^+FOXP3^+CD127^{low}$ is the most widely accepted phenotype for characterising nTregs.

Naturally occurring Tregs (nTregs)

Naturally occurring Tregs have the important function of preventing reactivity to both 'self' and 'non-self' antigens in an IL-2 dependent manner (Thornton, Donovan et al. 2004). IL-2 is necessary for proliferation and full activation of the responder T cells, however the mechanism by which Tregs inhibit IL-2 production is not clear. The inhibitory effects of nTregs are thought to involve both direct cell-to-cell contact and indirect (via APC presentation) mechanisms in an antigen specific manner. Human $CD4^+CD25^{high}$ T cells exhibit suppression of proliferation of $CD4^+CD25^-$ T cells in direct cell contact-

dependent manner or via the inhibition of DC function (e.g. via IL-10 or TGFβ production) (Jonuleit, Schmitt et al. 2001; Ito, Hanabuchi et al. 2008; Vignali, Collison et al. 2008). Conflicting studies report on the involvement of IL-10 and TGFβ in mediating the suppression by nTregs. Some studies have found blocking these cytokines did not affect the suppressive function of the nTreg. IL-10-deficient nTregs were found to be protective in the mice model of colitis, implying that IL-10 is not important in the anti-inflammatory activity of nTregs in this model (reviewed in (Wing, Fehervari et al. 2006)). Although cell-to-cell contact is required for suppression, this may not involve APCs (antigen presenting cells) as regulation is apparent in APC free systems (Thornton and Shevach 2000). However it appears that DCs may be important for the homeostasis of nTregs. Suppression of nTregs is reversed by the stimulation of DCs with CpG via TLR9 which is thought to be partly dependent on IL-6 production (Pasare and Medzhitov 2003). Interestingly both human and murine nTregs have been shown to express granzyme A or B after activation and are able to kill other cells in a perforin-dependent way (Grossman, Verbsky et al. 2004) although this is controversial and does not support the idea of reversible suppression of T cells. Once stimulated it is thought that nTregs can also have a bystander effect suppressing both $CD4^+$ and $CD8^+$ T cells responses non-specifically (Thornton and Shevach 2000).

In mice, adoptive transfer of $CD4^+$ T cells depleted of $CD25^+$ cells to athymic mice elicits a variety of autoimmune diseases, whereas co-transfer with $CD4^+CD25^+$ cells inhibited such disease development (Sakaguchi, Sakaguchi et al. 1995; Takahashi, Kuniyasu et al. 1998). Indeed the frequency and severity of autoimmune disease induction seems to correlate closely with the extent of the nTregs depletion (Ono, M *et al*, 2006).

Adaptive Tregs (aTregs or iTregs)

Adaptive Tregs are thought to be derived in the periphery from conventional T cells following antigen stimulation and are associated with production of the suppressive

cytokines IL-10 and TGFβ (Tr1 and Th3 T cell subsets respectively) and a lack of IL-2 (Groux, O'Garra et al. 1997; Barrat, Cua et al. 2002; O'Garra, Vieira et al. 2004; Nicolson, O'Neill et al. 2006; Wakkach, Augier et al. 2008). Both Tr1 and Th3 T cells are thought to control specific autoimmune diseases in various murine models including colitis (Groux, O'Garra et al. 1997) and experimental autoimmune encephalitis (EAE) respectively (Weiner 2001). Interestingly, TGFβ, in combination with IL-6, can induce Th17 pro-inflammatory T cells with opposing effects to aTregs which may be important in the control of infection (Bettelli, Carrier et al. 2006). FOXP3 is generally associated with nTregs, but it can also be induced in conventional peripheral $CD4^+$ T cells in situations of low doses of antigen (Kretschmer, Apostolou et al. 2005) making the distinction between nTregs and aTregs difficult.

In addition to the Tregs described above, there is evidence for other regulatory T cells including $CD8^+$ suppressor T cells that can secrete IL-10 or TGFβ (Haynes, Vanderlugt et al. 2000; Garba, Pilcher et al. 2002), γδ T cells that suppress anti-tumour activity of CTLs and NK cells (Seo, Tokura et al. 1999), and NKT cells that secrete IL-10 (Sonoda, Faunce et al. 2001).

1.8 ROLE OF TREGS DURING INFECTION

Controlling infection requires recognition of the pathogen and elimination by a series of controlled immune responses while limiting collateral damage from exuberant immune reactivity. Ideally, this would lead to immunity against subsequent challenge with the infection. At the same time micro-organisms would benefit from incomplete elimination for successful transmission. It is evident that Tregs play a significant regulatory role in control of the host pro-inflammatory response, thus limiting collateral damage, as mentioned in the previous sections. Many intracellular pathogens including leishmaniasis, malaria, HIV, TB and dengue fever (Belkaid, Piccirillo et al. 2002; Aandahl,

Michaelsson et al. 2004; Hisaeda, Maekawa et al. 2004; Mills 2004; Luhn, Simmons et al. 2007; Scott-Browne, Shafiani et al. 2007) (reviewed in (Belkaid 2007)) have all been shown to be controlled by Tregs. Most of this work has been in mice, but more recent human studies suggest similar regulatory roles in human infections. Increased Tregs during infection is generally associated with suppressive cytokine production (IL-10 and TGFβ), higher pathogen loads and persistence leading to chronic infection, whilst benefits for the host include reduced immunopathology from uncontrolled pro-inflammatory responses and prevention of autoimmunity (Boussiotis, Tsai et al. 2000; McGuirk, McCann et al. 2002; Couper, Blount et al. 2008).

In mice infected with herpes simplex virus (HSV), nTregs have been shown to protect against the development of virus-induced inflammatory lesions (Suvas, Azkur et al. 2004). Chronic infection with *Schistoma mansoni* also illustrates the protective role of nTregs against immunopathology, as their removal increases damage to the liver (Hesse, Piccirillo et al. 2004). Tregs have been implicated in the immunological hyporesponsiveness associated with other chronic infections in humans including cytomegalovirus (CMV), hepatitis B virus (HBV), HIV, *Chlamydia trachomatis*, and TB (reviewed in (Belkaid 2007). The removal of which can induce protection from some pathogens. *Plasmodium yoelii*, a parasite strain responsible for lethal rodent malaria, is eradicated from the body after removal of nTregs (Hisaeda, Maekawa et al. 2004). In humans the removal of $CD4^+CD25^+$Tregs enhances *in vitro* proliferation and IFNγ production in response to malaria antigens in blood from patients infected with *Plasmodium falciparum* (Walther, Tongren et al. 2005).

The role of Tregs in enhancing chronicity of infections is nicely illustrated in the *Leishmania major* murine model. Antigen specific nTregs ($CD4^+CD25^+$) accumulate in the dermis at sites of infection with *L. major*. These suppress IFNγ production and the ability of effector T cells to eliminate the parasite from the host using IL-10-dependent and IL-10-independent mechanisms (Belkaid, Piccirillo et al. 2002). Re-infection at a distal site leads

to immunity at the second site, but re-activation at the primary site. If anti-CD25 antibody is given intravenously prior to challenge, immunity is achieved in both sites, suggesting that re-activation is a result of the local immunosuppressive effect of Tregs at the original site (Mendez, Reckling et al. 2004). Functional Tregs ($CD4^+CD25^+CTLA^-4^+FOXP3^+GITR^+$) were also found in the skin of human visceral leishmaniasis patients that were able to produce large amounts of IL-10 and TGFβ, and suppress phytohaemagglutinin (PHA)-induced proliferation (Campanelli, Roselino et al. 2006).

In humans, HIV-specific nTregs from HIV infected individuals showed strong suppressive function *in vitro* that correlated with lower viraemia in plasma and higher $CD4^+$ to $CD8^+$ ratios (Kinter, Hennessey et al. 2004). In addition, individuals that are infected with hepatitis C virus (HCV) have a higher number of nTregs in the blood compared with uninfected individuals and depletion of these cells enhances antigen-specific $CD8^+$ T cells responses *in vitro* (Sugimoto, Ikeda et al. 2003). The numbers of nTregs inversely correlate with liver damage suggesting the role they play in controlling inflammatory responses and liver damage (Bolacchi, Sinistro et al. 2006).

Naturally occurring Tregs can produce IL-10, as observed in the *L. major* model, but generally Tr1 aTregs are the source of IL-10. These develop from conventional T cells in certain conditions such as exposure to immature APCs, repeated exposure to antigen, or the presence of IL-10 itself. One of the earlier studies of antigen-specific IL-10-producing Tr1 cells, was in response to malaria antigens. Selected alternative peptide ligands (APL) of the immunodominant CD4 T cell epitope downregulated IFNγ and proliferation, by preferential induction of IL-10 suggesting induction of Tr1 suppressor cells (Plebanski, Flanagan et al. 1999). Inducible antigen-specific Tr1 clones were generated in mice infected with *B. pertussis* (McGuirk, McCann et al. 2002), and isolated from humans with HCV (MacDonald, Duffy et al. 2002). The mouse study showed suppression of Th1 by Tr1-cell clones specific for bacterial antigens, whereas the human study indirectly

supported a role for IL-10 producing T cells by demonstrating increased IFNγ production *in vitro* after addition of neutralising IL-10 antibodies.

FOXP3 can be induced by TGFβ producing T cells, adding another level of complexity to the definitions of the Treg subsets. After experimental malaria infection of human volunteers, enhanced $TGF\beta^+$ and $FOXP3^+$Tregs were detected in the peripheral blood that correlated with faster parasite growth rates (Walther, Tongren et al. 2005). It has been proposed that the additional subset of induced $FOXP3^+$ Tregs may be required in the gut to regulate between tolerance to commensal bacteria and immunity to invading pathogens (Belkaid 2007). This is supported by the observation that the gut is the main site where these converted cells have been found. After oral exposure to antigen (OVA), DCs from the lamina propria of the small intestine of lymphopenic mice were found to promote high levels of Treg conversion relative to lymphoid derived DCs. This was dependent on retinoic acid (RA; a vitamin A metabolite highly expressed in the GALT) in the presence of TGFβ (Sun, Hall et al. 2007). This peripheral source of Tregs could be particularly important in long-term chronic conditions such as *M.tb* since thymic output is limited in an older age group and the nTreg supply is likely to decline.

1.8.1 Tregs and TB

Both nTregs and aTregs have a role in TB disease. IL-10 is involved in tuberculin skin test (TST) anergy, which is associated with poor prognosis by unknown mechanisms (Boussiotis, Tsai et al. 2000; Delgado, Tsai et al. 2002). IL-10 specific neutralising antibodies increase PPD-specific IFNγ production by T cells from non-responders *in vitro* suggesting that Tr1 cells mediate T cell suppression in TB patients (Boussiotis, Tsai et al. 2000). It is also clear that in mice foot pad swelling of a DTH response to PPD was reduced by abrogation of IL-10 (Nadler, Luo et al. 2003). TGFβ has been shown to be secreted by *M.tb* infected macrophages (Othieno, Hirsch et al. 1999). Naturally occurring

Tregs are also upregulated in patients with tuberculosis (Guyot-Revol, Innes et al. 2006; Ribeiro-Rodrigues, Resende Co et al. 2006; Chen, Zhou et al. 2007; Hougardy, Verscheure et al. 2007; Li, Lao et al. 2007) and depress immune responses to the protective heparin-binding haemagglutinin antigen (HBHA). Interestingly the latter study showed that this suppression was independent of IL-10 and TGFβ (Hougardy, Place et al. 2007). Upregulated *FOXP3* expression has also been associated with TB disease (Guyot-Revol, Innes et al. 2006; Burl, Hill et al. 2007). In one study *M.tb* latently infected subjects (PPD^+, TST^+, EC^+) had a significantly lower FOXP3 mRNA levels than in both the controls (PPD^+, TST^-, EC^-) and in the TB cases. It was speculated that the Tregs sequestered to the lungs during infection but active disease led to increased Tregs and migration in peripheral blood (Burl, Hill et al. 2007). This is supported by studies in mice and monkeys that have shown that Tregs proliferate and accumulate at sites of *M.tb* infection (Scott-Browne, Shafiani et al. 2007)(Green, A *et al*, personal communication).

Collectively these findings particularly support a role for IL-10 producing Tr1 T cells in the control and chronicity of mycobacterial infection and a possible role for $FOXP3^+$Tregs.

1.9 VACCINATION AND TREGS

It is clear that regulatory T cells can control secondary responses to infections in both specific and non-specific manners. This would suggest that responses to vaccination could be affected by memory Tregs induced by vaccine specific or different microbes. In mice studies using a vaccine against *L. monocytogenes*, pre-existing nTregs restricted the magnitude of pathogen-specific $CD8^+$ T cell responses upon secondary challenge with the bacterium or vaccine (Kursar, Bonhagen et al. 2002). Similarly depletion of nTregs using anti-CD25 antibody (resulted in 44 – 65% reduction in $CD4^+FOXP^+$ T cells) prior to vaccination with MVA-CSP malaria vaccine in mice enhanced IFNγ responses, increased parasite control and more durable immunity (at least to 100 days when the effects of anti-

CD25 would be eliminated) compared to vaccine alone (Moore, Gallimore et al. 2005). Interestingly, if vaccination induces a suboptimal response it can increase the likelihood of inducing Tregs, and on re-challenge these Tregs reduce the protective response. This has been clearly shown with the use of the *Leishmania* antigen LACK in mice vaccination trials. When used in conjunction with an adjuvant, protection was achieved, but the absence of adjuvant favoured the emergence of IL-10-producing regulatory T cells which predicted vaccine failure (Stober, Lange et al. 2005). However the induction of the Tregs can also prevent immunopathology associated with vaccination. Thus, vaccination against *B. Burgdorferi* induces an osteoarthropathy which is reduced with the induction of nTregs (Nardelli, Burchill et al. 2004).

Early studies have found suppressor T cells induced by BCG vaccination in rats, that hamper efficient immunity upon revaccination but these were not defined as Tregs until later (Ha, Waksman et al. 1974). In addition, cell mediated immunity to BCG infection in mice is increased in IL-10 deficient mice, and these mice eliminate the bacteria more quickly than wild type mice (Jacobs, Fick et al. 2002). Furthermore, in mice, the level of Tregs prior to vaccination altered the Th1 response to the BCG vaccine, but Tregs were not thought to be involved in protection from BCG vaccination (Quinn, Rich et al. 2008). Additional studies in mice (Quinn, McHugh et al. 2006; Li and Shen 2009) and 10 week old human infants (Hanekom 2005) have shown an upregulation of FOXP3 Tregs but the role of Tregs in human BCG vaccination is not clear.

1.10 HYGIENE HYPOTHESIS AND TREGS

It has been postulated that Tregs play a role in the controversial 'hygiene hypothesis'. Continuous exposure to harmless mycobacteria could lead to increased background levels of activated Tregs which have a non-specific bystander effect or dampen responses against 'self' such as in the gut. The increased numbers of regulatory DCs lead to increased Tregs against 'self', gut associated antigens and allergens

downregulating autoimmunity, inflammatory bowel diseases and allergies (Rook, Hamelmann et al. 2007).

The interaction between dendritic cell and mycobacteria can occur via pathogen recognition receptors, such as the TLRs. Evidence for TLR modulation of allergy comes from experiments where administration of LPS (TLR4 agonist) in mice prior to allergen sensitisation suppressed IgE production, development of airway eosinophilia and Th2 responses (Gerhold, Blumchen et al. 2002). In addition, polymorphisms of several TLR molecules have been associated with increased allergy (reviewed in (Garn and Renz 2007) and (Yang, Fong et al. 2006). Interestingly Tregs could also be modulated by PAMPs including those derived from *M.tb* (Caramalho, Lopes-Carvalho et al. 2003).

It is likely that cytokines play a role in polarising DCs and controlling allergy. Decreased IL-4 and IL-5 production from Th2 $CD4^+$ T cells and increased IFNγ from Th1 T cells is associated with successful venom-specific immunotherapy (VIT) which induces tolerance to insect stings (Durham and Till 1998). It has also been found that IL-10 and/or TGFβ Tregs are generated during the early stages of allergen-specific immunotherapy suggesting the involvement of Tregs in reducing allergy (Jutel, Akdis et al. 2003) discussed in (Jutel, Akdis et al. 2005)). The role of IL-10 has shown conflicting results with IL-10 polymorphisms found in those with asthma in addition to lower levels of IL-10 in the bronchoalveolar lavage fluid (Borish, Aarons et al. 1996; Lyon, Lange et al. 2004). IL-10 is a potent suppressor of IgE and can skew the specific response from IgE to an IgG_4 dominated phenotype (Jutel, Akdis et al. 2005). However atopic dermatitis has been associated with increased IL-10 and IL-13 in the peripheral blood (Lee, Lee et al. 2000; Aleksza, Lukacs et al. 2002; Szegedi, Barath et al. 2009). Seneviratne *et al* showed that although IL-10 was induced by individuals with mild atopic dermatitis those with the more severe form had lower frequencies of IL-10 producing allergen-specific circulating $CD4^+$ T cells compared to non atopic controls. This was accompanied by higher frequencies of TNFα, IL-4, IL-5 and IL-13 (Seneviratne, Jones et al. 2006). T cell involvement and in

particular the role of Th2 cytokines in atopic dermatitis are reviewed in Ogg, G (Ogg 2009).

BCG vaccination inhibits a *de novo* allergic inflammatory response in a mouse model of asthma, in association with increased $CD4^+CD25^+$ T cells and FOXP3 expression and increased IL-10 and TGFβ expression (Li and Shen 2009), suggesting that Tregs induced by mycobacteria were responsible for suppression of this allergic disorder. Interestingly, IPEX patients that have dysfunctional FOXP3 expression are predisposed to dermatitis and food allergies linking the presence of Tregs to decreased allergy (Torgerson, Linane et al. 2007; Wan and Flavell 2007). BCG has also been shown to suppress Th2 responses by altering the function of allergen-activated DCs to induce IFNγ and IL-10 producing Th1 T cells (Yokoi, Amakawa et al. 2008). In the case of autoimmunity, a study in Argentina found that multiple sclerosis (MS) patients who developed parasite infections had fewer MS exacerbations than those that did not. Interestingly the parasite infected patients had an induction of Tregs ($CD4^+CD25^{high}FOXP3^+$) specific for myelin basic protein (MBP) (Correale and Farez 2007).

The age of exposure to mycobacteria appears to be significant in its ability to reduce atopy, since early life or even prenatal exposure result in the most pronounced effects. In a murine model, LPS applied to pregnant mice reduced the allergic response to OVA in the offspring with reduced levels of IgE, IgG1 and IL-5 and IL-13 Th2 cytokines (Blumer, Herz et al. 2005). There are limited humans studies, but examination of intestinal flora of children growing up in different allergy risk areas has shown different bacteria colonising the gut in the first year of life (Majamaa and Isolauri 1997). Administration of *Lactobacillus rhamnosus GG* (LGG)-containing probiotic formula given to neonates was shown to reduce the development of cow's milk allergy (Majamaa and Isolauri 1997).

Although there is accumulating evidence to support the hygiene hypothesis, other studies fail to show these associations and suggest that the differences are due to host effects rather than causal effects between infection and atopy. Obhara and Bardin discuss

the body of evidence relating to BCG vaccination and atopy in human studies. Their analysis found that 10 out of 23 published epidemiological studies showed an inverse relationship between BCG and atopic disease, but no relationship with geographical location (Obihara and Bardin 2008). In The Gambia no relationship between tuberculin skin test positivity (a measure of either pathogenic or non-pathogenic mycobacterial exposure or BCG vaccination in these children) and atopy (measured by response to at least one common allergen) was found (Ota, van der Sande et al. 2003). By contrast children living in more rural areas of the Gambia were more likely to be atopic (43% positive for at least one allergen tested) than in the urban areas (22%) (Ota, van der Sande et al. 2003). It was suggested that intense exposure to dust in these areas might lead to high exposure to the most common allergen causing asthma, the house dust mite. Important factors that may explain differences in the outcomes between these studies include heterogeneity in study design, variability in immunity to BCG, influence of other mycobacteria including dose and persistence of NTM, and simultaneous BCG and allergen exposure (Obihara and Bardin 2008).

Taken together Tregs (both naturally occurring and adaptive) are increasingly described to be participating in most immune responses in variable proportions to either the advantage of the host or pathogen. It is likely that the magnitude, and the timing of Treg induction are crucial in determining their benefit or otherwise to the host.

1.11 INFANT IMMUNOLOGY

Studying the development of infant immunity in humans is difficult for experimental, practical and ethical reasons and therefore many studies are based on the more easily available cord blood or detailed mouse studies. Immune development in mice and humans is similar in the sequence of events however the kinetics are quite different; in humans immunocompetent T cells are developed by mid-gestation and in mice maturity of

T cells occurs postnatally (Fadel and Sarzotti 2000). The neonatal period in humans is restricted to the first 28 days of life, after which it is defined as an infant; whereas for mice this period is less well defined and is described as the first 1-7 days of life (Fadel and Sarzotti 2000; Siegrist 2001). Since the 1980's scientists have worked on designing mouse models of the human immune system, often termed the 'humanised mouse'. Although there are now many sophisticated humanised mouse models available (Legrand, Weijer et al. 2006) they still do not provide a realistic *in vivo* representation of immune development in humans, where environmental and genetic factors often play an important role. With this in mind, alongside the high levels of infant mortality worldwide, and the increasing number of vaccines that the infant's immune system needs to deal with, it is imperative to study immune responses in human populations, particularly in high disease burden settings.

1.10.1 Maternal transfer of immunity

During the first few months of life maternal antibodies that have transferred from the placenta during gestation protect the infant from micro-organisms to which the mother has immunological memory. However lymphocytes are not transferred in this way and therefore infants are susceptible to infectious diseases that require T cell mediated protection, in particular intracellular pathogens.

There is very little known about what antigens cross the placental barrier, but malaria studies have isolated parasites in cord blood and malaria specific IgM antibodies, an Ig isotype that does not cross the placenta (Malhotra, Ouma et al. 1997; King, Malhotra et al. 2002; Brustoski, Kramer et al. 2005; Brustoski, Moller et al. 2006; Malhotra, Mungai et al. 2006). Therefore it is not inconceivable that mycobacterial antigens may also cross the placental barrier, as indicated in a Kenyan study that examined *in utero* exposure to helminth and mycobacterial antigens (Malhotra, Ouma et al. 1997). In this study, cord blood from 30% of neonates responded to PPD after 3 days of culture producing 70 –

2,000 pg/ml of IFNγ. Helminth specific responses were also found in cord blood suggesting prenatal exposure to helminths and mycobacteria may lead to tolerance or altered foetal immunity (Malhotra, Ouma et al. 1997). Transplacental or *in utero* vertical transmission has also been demonstrated in various animal models of mycobacterial infection (Sweeney, Whitlock et al. 1992; Judge, Kyriazakis et al. 2006; Alinovi, Wu et al. 2009; Singh, Sohal et al. 2009), although it is possible that the placental environment is quite different in cattle, goats and rabbits compared to humans.

Breast feeding can reduce the frequency and severity of gastrointestinal and respiratory infections in infants mainly due to the transfer of maternal humoral immunity in breast milk. Breast feeding can also enhance cell mediated responses to the mitogen PHA and tetanus toxoid up to 6 weeks of life (Stephens, Brenner et al. 1986). Interestingly, BCG responses were enhanced in breastfed infants vaccinated at birth, but not those vaccinated later than 1 month of life (Pabst, Godel et al. 1989). The extent of cell-mediated immunity transmitted through breast milk is unclear and several older studies give conflicting results. One study found PPD specific T cells in breast milk but no responses to PPD in cord blood suggesting the mycobacterial antigens from a PPD^+ mother did not cross the placental barrier (Schlesinger and Covelli 1977), whereas others have found responses to PPD in cord blood of children exposed to PPD positive mothers, but no difference between those that were breast fed and those that were not (Keller, Rodgriguez et al. 1987) implying transplacental transmission of mycobacterial antigens but not via breast milk.

1.10.2 Tolerance

Increased susceptibility of neonates to infections has been proposed to be a consequence of an immature immune system and induction of neonatal tolerance (Billingham, Brent et al. 1953). Mice injected with alloantigens at birth are rendered tolerant, but later in life the same encounter stimulates a strong Th1 immune response

(Billingham, Brent et al. 1953).

The process of T cell selection by clonal deletion occurs in the thymus to avoid reaction to 'self'. Those T cells with high affinity binding to 'self' antigens are removed by negative selection, while those with low affinity binding to antigens are eliminated by positive selection. The remaining T cells are released into the periphery and express a diverse repertoire of T cell receptors (TCRs) to foreign antigens (Burnet 1957; Sprent 1995). Following this principle, antigens introduced early in life activate their specific T cells, causing them to get activated at a time that the host immune system is eliminating self-reacting cells, therefore these activated T cells are deleted without a deposit of memory. When such a host encounters the same antigen there is an anergic tolerogenic response. For example, infection of neonatal mice with lymphocytic choriomeningitis virus (LCMV) results in tolerance to infection (Evans, Borrow et al. 1994). A decreased ability to respond to certain stimuli in early life was supported by many studies, but in the late 1990's it became clear that neonates (both mice and humans) had the ability to respond to specific stimuli at a magnitude comparable to adults, but with a great deal of variability ranging from non-responsiveness to fully mature function. This appears to depend on many factors including quantity of functional immune cells present, type of stimulus, antigenic dose and environment (Forsthuber, Yip et al. 1996; Adkins and Du 1998) (Marchant, Goetghebuer et al. 1999; Chipeta, Komada et al. 2000) (reviewed in (Adkins, Leclerc et al. 2004)).

1.10.3 Cord blood studies

A large extent of what is known about early life immunology in humans has come from cord blood studies since large volumes are easily available and collection is non-invasive. Cord blood is often studied as a source of antigen naïve T cells ($CD45RA^+/RO^-/CD62L^+$) but this may not be as robust as once thought. Results from comparative studies between cord blood and adult peripheral blood are often conflicting and make it difficult to

understand the true differences. Although the majority of T and B cells in cord blood are $CD45RA^+$ (Szabolcs, Park et al. 2003), Byrne *et al* found that at least 25% of T cells from the cord blood of premature foetuses were $CD45RO^+/RA^-$ on both $CD4^+$ and $CD8^+$ T cells. The $CD4^+$ population frequently expressed $CD25^+$ (IL-2R alpha) and were able to proliferate in response to exogenous IL-2, however, in contrast to adults, they were unresponsive to mitogenic anti-CD2 or anti-CD3 antibodies suggesting anergic properties (Byrne, Stankovic et al. 1994). This compared to another study which showed that, 48 hours after cord blood stimulation with anti-CD3 antibody, T cells expressed CD25 and proliferated in an IL-2 dependent manner. By 72 hours the cord blood T cells showed extensive apoptosis compared to adult cells, accompanied by increased CD95L expression although not mediated by the FasL pathway (Canto, Rodriguez-Sanchez et al. 2003).

This increased apoptosis along with increased proliferation compared to adults has been shown in several other studies of cord blood lymphocytes (Tu, Cheung et al. 2000; Yang, Hsu et al. 2001; Canto, Rodriguez-Sanchez et al. 2003; Szabolcs, Park et al. 2003; Kessel, Yehudai et al. 2006). Szabolcs *et al* found that the proliferating T cells still possessed a naïve phenotype ($CD45RA^+/RO^-$, $CD69^-$, $CD25^-$ and $HLA\text{-}DR^-$), with an increased absolute number of T, B and NK cells in cord blood. $CD8^+CD57^+CD28^-$ 'suppressor', $CD8^+CD45RA^+CD27^-$ 'cytotoxic' and the skin homing marker, cutaneous lymphocyte-associated antigen (CLA) were all absent among the T cells in cord blood (Szabolcs, Park et al. 2003). However, Keever *et al* found reduced numbers of NK cells and lower proliferative, stimulatory and cytotoxic activity for cord blood mononuclear cells compared to adult PBMC (Keever 1993). The progression of naïve T cells to effector cells is dependent on the survival of antigen specific T cells and their resistance to apoptosis. This process of T cell maturation in cord blood has been linked to insulin-like growth factor 1 (IGF-1) which enhances the PHA-induced $CD45RO^+$ T cells and reduces their susceptibility to apoptosis through IL-6 production (Tu, Cheung et al. 2000) (Law, Tu et al. 2008).

1.12 IMMUNITY TO INFECTIONS IN EARLY LIFE

In human disease, malaria infected children less than 5 years of age have diminished Th1 and Th2 malaria-specific responses compared to adults (Xainli, Baisor et al. 2002). However a study in Kenya showed similar responses to thrombospondin-related adhesive protein of *Plasmodium falciparum* (PfTRAP) peptide epitopes in both adults and children (Flanagan, Mwangi et al. 2003). In addition, adult level Th1 responses can be induced by BCG vaccination in neonates (Marchant, Goetghebuer et al. 1999; Vekemans, Amedei et al. 2001; Hussey, Watkins et al. 2002). Th1 and Th2 T responses to polyclonal TCR activation have also shown comparable profiles between cord blood and adults including IFNγ, IL-12, TNFα and IL-10 production.

1.11.1 Defects in adaptive responses in early life

Increased susceptibility to infection in neonates has been linked to a Th2 biased response and reduced cytokine production in response to inflammatory stimuli, rather than the inability of lymphocytes to proliferate (Krampera, Tavecchia et al. 2000; Smart and Kemp 2001). Production of IL-12 in neonates has been shown to be defective, a cytokine that plays a central role in Th1 differentiation and production of IFNγ (Goriely, Vincart et al. 2001). Another study found changes in transcriptional regulation of the IFNγ promoter caused reduced IFNγ production from CD4 T cells, but not CD8 T cells, in neonatal naïve cells (White, Watt et al. 2002). Despite the T cell tolerance and anergy described in neonates characterised by Th2 development and lack of cytotoxic T cells (CTL), strong CTL function has been detected in human infants with the congenital CMV infection, demonstrating that mature CTL activity is possible in early life (Marchant, Appay et al. 2003). Interesting studies in mice have shown that T cells from neonatal mice that are susceptible to *Pneumocystis carnii*-induced pneumonia can resolve infection when transferred into adult mice, suggesting the defects are not inherent within the T cell but

may be related to the cytokine milieu (Qureshi and Garvy 2001). The mechanism of the Th2 skewing observed in many neonatal responses may be explained by increased susceptibility of neonatal memory T cells to apoptosis after re-exposure to antigen (Li, Lee et al. 2004).

Differences between neonates and adults have been shown for B cell function (reviewed in (Adkins, Leclerc et al. 2004)). Neonatal antibody responses are delayed in onset, reach lower peak levels, are of shorter duration, differ in IgG2 distribution in humans and are of lower affinity and reduced heterogeneity than adult responses. In humans, the reduced antibody responses are thought to be partially due to the presence of maternal antibodies, and may contribute to the higher vaccine failure rates observed in young infants. This reduced antibody function is not associated with intrinsic B cell defects, but is likely to be due to limited development of the B cell compartments and/ or colonisation of plasma cells in the bone marrow (Adkins, Leclerc et al. 2004).

1.11.2 Defects in innate responses early in life

Less is known about the neonatal innate immune response, but several studies have shown that neonatal dendritic cells have an inability to deliver signals required for the polarisation of immune responses towards a Th1 pro-inflammatory protective response (Trivedi, HayGlass et al. 1997; Delespesse, Yang et al. 1998). IL-12 derived from DCs plays a role in both the innate and the adaptive response. It has been observed that neonatal dendritic cells do not respond well to TLRs, leading to defective production of IL-12 (Levy, Zarember et al. 2004; Krumbiegel, Zepp et al. 2007). However, it is interesting to note that TLR8 and the dimer TLR7/8 can induce a mature response in cord blood with production of TNFα and IL-12/IL-23 as well upregulation of CD40 by neonatal DCs (reviewed in (Levy 2007). Increased B cell production of IL-10 has also been associated with reduced IL-12 production from dendritic cells, and may contribute to this defect (Sun,

Deriaud et al. 2005).

It is also evident that neonates have a reduced number of granulocyte and monocyte progenitor cells which can lead to a reduction in neutrophil numbers during stress conditions. The neutrophils have also been shown to be functionally defective with an inability to migrate to sites of inflammation and contribute to an overall 'normal' immune response (Carr, R 2000). Defects in neonatal immune cells may also be controlled by the physiological environment. Human neonatal cord blood mononuclear cells contain increased concentrations of cyclic adenosine monophosphate (cAMP) compared to adults which can lead to an inhibition of IFNγ and IL-12 while enhancement of cytokines such as IL-6, IL-10 and IL-23 (Schnurr, Toy et al. 2005). By contrast, certain cytokines such as IL-6, IL-10 and IL-23 have all been shown to be produced in greater quantities from neonatal monocytes and antigen presenting cells (APCs) than in adults (Angelone, Wessels et al. 2006) (Chelvarajan, Collins et al. 2004; Vanden Eijnden, Goriely et al. 2006).

It is therefore evident that neonatal responses in cord blood are different to those of adults, often leading to poorer CD4 and CD8 T cell function as a result of suboptimal antigen presenting cell-T cell interactions. However in certain conditions adult-like T cell responses can be induced thus overcoming these deficiencies.

1.13 VACCINATION IN EARLY LIFE

Due to the above deficiencies in the maturity of the immune system, several doses of vaccines are often needed to be given to young children to achieve protective levels of antibodies. The earlier in life infants are vaccinated, the poorer and more short-lived their antibody responses are, irrespective of the protection from maternal antibodies (reviewed in (Siegrist 2001)). However, vaccinating later in childhood exposes the child to a window of susceptibility to childhood infections at a time when the child is most vulnerable. Induction of significant primary IgG responses to vaccines can occur at >2 months of age,

but persistence and adult-like levels are normally not possible until >12 months of age. Furthermore, the protein and polysaccharide based vaccines are generally unable to induce protective responses until >18 months of age (Siegrist 2001). Lower IFNγ responses are associated with higher antigen specific T cell derived Th2 responses, weaker induction of IgG2 antibodies and limited cytotoxic T cells following early life immunisation, however most of this work is in mice (reviewed in (Siegrist 2001)). Despite these shortcomings, it is still important to prime the immune response at an early age. Oral polio vaccine (OPV) given at birth elicits a weak primary response in humans, but an increase in antibody levels to the second dose compared with unprimed infants (Dong, Hu et al. 1986). Interestingly in mice, neonatal Th1 responses are generally transient with a largely Th2 secondary memory responses. It has been suggested that these initial Th1 T cells are more susceptible to apoptosis (Singh, Hahn et al. 1996; Adkins and Du 1998).

Despite this, Th1 responses to PPD similar to those seen in adults were observed in response to BCG vaccination in Gambian infants at birth (Marchant, Goetghebuer et al. 1999; Vekemans, Amedei et al. 2001), and these remained present at 1 year although at a lower level than 2 months after vaccination (Marchant, Goetghebuer et al. 1999). This is in contrast to unvaccinated individuals whose primary responses to PPD were predominantly Th2 biased (Marchant, Goetghebuer et al. 1999; Vekemans, Amedei et al. 2001). In addition, BCG vaccination also induces a cytotoxic CD8 response in neonates that, although not as strong as the CD4 response, may also contribute to protection of BCG (Hussey, Watkins et al. 2002; Murray, Mansoor et al. 2006). Recent studies in South Africa showed *in vitro* IL-12, IL-10 and IFNγ production in response to BCG was predominantly from innate cells at birth, including monocytes and NK cells, but was T cell derived by 13 weeks after BCG vaccination suggesting an innate response may play a role in protection against mycobacterial infections in early life (Watkins, Semple et al. 2008).

The extent to which maternal antibodies influence vaccine responses is not clear with some vaccines being inhibited, but others showing no influence at all, but induction of

T cell responses largely remain unaffected by these passively transferred antibodies (reviewed in (Siegrist 2003).

1.14 TREGS IN EARLY LIFE IMMUNITY

It has been long been known that neonatal thymectomy in mice leads to an increased incidence of autoimmune disease which can be prevented by infusion of adult Tregs (Suri-Payer, Amar et al. 1998). T cells with regulatory or suppressive phenotypes ($CD4^+CD25^{high}$) have been identified during foetal development (Byrne, Stankovic et al. 1994; Cupedo, Nagasawa et al. 2005; Darrasse-Jeze, Klatzmann et al. 2006; Michaelsson, Mold et al. 2006) and are thought to be essential in early life as part of immune tolerance to self antigens. Interestingly $CD4^+CD25^{high}$ Tregs appear to be detected at greater frequencies during gestation than at birth suggesting a role in intrauterine immune control (Takahata, Nomura et al. 2004). Interestingly a recent study showed that the transfer of maternal cells across the placenta induced $CD4^+CD25^{high}FOXP3^+$ Tregs that suppressed foetal anti-maternal immunity, and persisted until at least early childhood (Mold, Michaelsson et al. 2008).

There are conflicting studies regarding the levels of Tregs in cord blood which may in part be due to the definitions used and assays performed. Many studies have suggested umbilical cord blood (UCB) has a greater percentage of $CD4^+CD25^{high}$ (5.1%) cells than adults (1.4%)(Wing, Ekmark et al. 2002) but this is in contrast to several other studies where similar numbers of $CD4^+CD25^+$Tregs (or $CD4^+CD127^{low}CD25^+$) were observed in cord blood (Takahata, Nomura et al. 2004; Santner-Nanan, Seddiki et al. 2008). However there is generally agreement that cord blood Tregs are of a more naïve phenotype ($CD45RA^+$) compared to adults (Wing, Ekmark et al. 2002; Takahata, Nomura et al. 2004). The phenotype of cord blood $CD4^+CD25^{++}$ Tregs from Wing *et al*'s study were predominantly $CD69^-$, $CD62L^{bright}$, $CD38^{bright}$, $CD152^+$ (CTLA-4) and $CD122^+$, with half the population being $CD45RA^+$. In contrast only 10% of adult Tregs were $CD45RA^+$. It

was proposed that naturally occurring Tregs leave the thymus in a naïve state and become activated in the periphery (Wing, Ekmark et al. 2002).

Functional Tregs have been isolated in the neonate (Ng, Duggan et al. 2001; Wing, Ekmark et al. 2002; Takahata, Nomura et al. 2004; Godfrey, Spoden et al. 2005), where their suppressive function is equivalent to the suppressive activity of adult $CD4^+CD25^+$ Tregs. It is most likely that in addition to controlling responses to self, these play a key role in the development of immune responses to foreign antigens and pathogens.

In addition to $CD4^+$ Tregs, recent reports implicate key roles for $CD8^+$ Tregs in neonatal life. CD8 Tregs have been found to down regulate Th2-induced pathologies, escaping neonatal tolerisation (Field, Caccavelli et al. 2003). The role of $CD8^+$ Tregs in response to vaccines is not yet known.

Control of the immune response is vital for the maintenance of health. Regulatory T cells are likely to be involved in many aspects of this control including responses to infections. It is likely that similar processes are involved in memory responses to vaccination and it is therefore important to consider the contribution of Tregs to protection by vaccination, especially in young children who receive numerous vaccines in early life and may have different immune responses than adults.

1.15 HYPOTHESIS

It was proposed that exposure to NTM early in life induces regulatory T cells that attenuate responses to BCG vaccination, and that this might account for the poorer immunogenicity of BCG vaccine in countries with high levels of NTM in the environment, as illustrated in Figure 1.3.

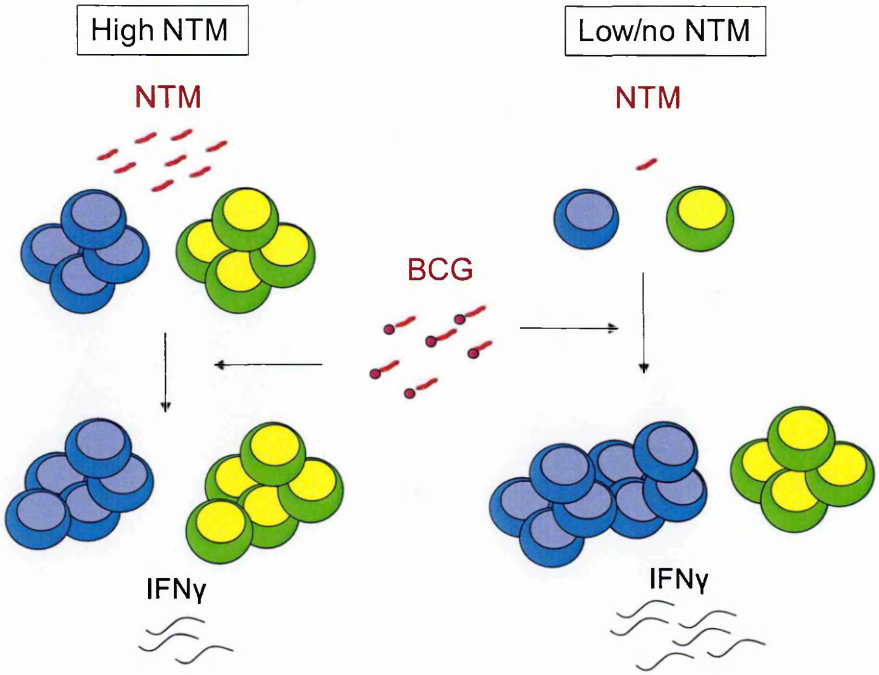


Figure 1.3: Proposed model of BCG vaccination with respect to non-tuberculous mycobacteria (NTM) exposure. *In areas of high exposure to NTM memory T cells specific for mycobacterial antigens are induced particularly regulatory T cells that would control the response to NTM specific antigens. On vaccination with BCG, that contains many similar antigens to NTM, a reduction in the pro-inflammatory (IFNγ) protective response of the vaccine is observed in areas with previous high exposure to NTM compared to that from a low exposed setting. It is hypothesised that is this is due to the level of mycobacterial specific Tregs that are present prior to vaccination. Blue cells represent effector memory T cells and green/yellow cells represent memory Tregs.*

CHAPTER 2

Materials and Methods

2.1 STUDY DESIGN

The study was approved by the MRC Scientific Co-ordinating Committee (SCC), the Gambian/MRC Research Ethics Committee and the London School of Hygiene and Tropical Medicine Research Ethics Committee.

Neonates were recruited at birth from the Sukuta Hospital with parental consent, and randomised into two groups according to BCG vaccination schedule;

- Group 1 were vaccinated with BCG at birth (or within 1 week)
- Group 2 were BCG vaccinated at 4½ months of age

Live, attenuated BCG vaccine (BCG Moscow strain (Mostowy, Tsolaki et al. 2003), Serum Institute of India Ltd, India, donated by UNICEF) was reconstituted using the accompanying vial of sodium chloride as per manufacturer's instructions and 50 µL was injected intradermally into the deltoid region of the left arm (each 0.1 mL contained 1 - 33 x 10^5 CFU) (Figure 2.1A). This BCG Moscow strain was typed by Dr Peter Keller, Institute of Medical Microbiology, IMM, Zurich, Switzerland. According to phylogenic analyses BCG Russia is an ancient strain most closely related to parental *M.bovis* (Keller, Bottger et al. 2008).

A B

Figure 2.1: Group 1 study participant being vaccinated with BCG (A) and being weighed as part of the monthly follow up (B)

All other vaccines were administered according to the National Expanded Programme of Immunisation (EPI) schedule (Table 2.1) by MRC field worker nurses in order to ensure venesection for this study occurred at least 2 weeks post vaccination.

At birth	1 month	2 months	3 months	4 months	9 months	>12 months
BCG						
Hepatitis B		Hepatitis B		Hepatitis B		
OPV	OPV	OPV	OPV		OPV (or later)	OPV (18 m)
		DTwP	DTwP	DTwP		DTwP
		Hib	Hib	Hib		Hib (16 m)
					Measles	
					Yellow fever	

BCG = Bacillus Guerin Calmette
OPV = oral polio vaccine
DTwP = Diphtheria, tetanus, whole cell pertussis combined vaccine
Hib = Haemophilis influenza type B

Table 2.1: Extended Programme of Immunisation (EPI) recommended schedule for

The randomisation was stratified for every 20 subjects. HIV status of the participants was not assessed; previous reports from The Gambia have found 2.4% HIV infection in adults (aged 15 – 49) and an estimated 1,200 children (aged 0 – 14 years, 0.1% of population) living with HIV (UNICEF 2005) suggesting that it is unlikely that any of the children in this study would be HIV^+. The study subjects were healthy babies, matched by age and socio-economic background, predominantly Mandinka in ethnicity and equal numbers were recruited through wet and dry seasons from June 2006 to August 2007.

Exclusion criteria included:

1. Birth weight < 2.5kg
2. Twins (to avoid genetic bias)
3. Any symptoms of intercurrent infection
4. Exposure to TB within their household or close contact with a TB case at time of recruitment
5. Any likelihood of long term travel out of the area within the 9 months of the study period.

As first contact with pregnant mothers is often on arrival at the maternity clinic during labour, verbal consent was taken in order to collect the cord blood at birth. Written consent was then obtained after birth when the fieldworker was able to discuss the details of the study with the mother. Only after written consent was obtained, were the cord blood samples processed in the laboratory. If the mother declined to participate in the study then the cord blood samples were destroyed at the clinic.

Blood and urine samples were collected at birth, 4½- and 9- months of age. Preliminary analyses demonstrated that optimal results from laboratory assays required that

the blood arrived within 6 hours of collection (data not shown). Every subject was followed up within the first week of life and then every month for the entire study. At each month, a TB questionnaire was completed to assess TB exposure of the subjects, and a routine health and morbidity questionnaire was completed to record anthropometric data (Figure 2.1B). At 9 months of age all subjects automatically became part of the Sukuta birth cohort and are currently being followed up, on a regular basis to obtain anthropometric and clinical data, for 4 years of life.

A copy of the information sheet provided to the mothers (or translated by the fieldworkers for the mothers), the consent form and the TB information sheet and questionnaire are all included as Appendices (Appendix I - IV).

2.2 TUBERCULIN SKIN TEST (TST)

Reactivity to the TST was assessed at 4½ months of age in all subjects. 0.1 mL of 2 T.U. (tuberculin units) RT23 SSI (Statens Serum Institute, Denmark) was injected intradermally into the mid flexor surface of the left forearm of the subject by a trained clinician or fieldworker. A definite white bleb was raised at the needle point, about 10 mm in diameter, which disappeared within 10 minutes. The induration was measured 48 - 72 hours later by two independent fieldworkers. A positive induration was recorded as recommended by SSI; the average length and width of the raised area of induration (evaluated by rolling the point of a pen across the arm and marking when it reaches resistance).

In The Gambia, it was assumed that a TST of 5 - 10 mm at 4½ months of age would be due to:

1. BCG vaccination, or
2. Exposure to NTM

Exposure to *M.tb* was suspected if the TST >10 mm. These children were followed up intensively to assess TB exposure, thus it was expected that exposure would be minimal.

If TB exposure was suspected from the skin test results or the TB questionnaire, a clinician would visit the compound of the subject and assess any likely exposure to a TB case within the compound. A skin test was also carried out on all members of the compound. Within the first 4½ months of life, any subject from the delayed vaccine group, thought to be exposed to TB was withdrawn from the study, screened for clinical evidence of TB and immediately given BCG vaccination if appropriate. Any subject or member of their compound, suspected to have TB, was referred to the local TB clinic for further analysis and treatment if required.

As an extension to this study, further ethics approval was granted to perform an additional TST at 20 - 28 months of age (Appendices V and VI). Interim analysis will be presented within this thesis. When visiting the children at this time point a T-shirt designed by a colleague (Dr. Matthew Cotten) was donated to each child to thank them for participating in the study (Figure 2.2).

Figure 2.2: Design for T-shirts given to all participants and staff involved in the study to thank them for their contributions (copyright Dr. Matthew Cotten)

2.3 SAMPLE COLLECTION

2.3.1 Cord blood collection

Immediately following delivery, before the placenta is separated, the umbilical cord was cleaned with alcohol and cotton and let to dry. A sterile prepared needle and syringe containing 7.5 Units of heparin per mL blood (sodium salt from porcine intestinal mucosa, Sigma-Aldrich, Poole, UK) was used to puncture a vein in the cord. Between 10 - 50 mL of foetal cord blood was collected by a trained nurse or fieldworker and mixed well before being transferred into a sterile 50 mL conical tube and stored into the fridge prior to transportation to the laboratories in Fajara (within 6 hrs of collection).

2.3.2 4½- and 9-month blood collection

Venous blood samples (1 mL/kg (max 5 mL)) were collected by trained fieldworkers from infants at 4½-, and 9- months using a 21 gauge needle and inserted into the back of the hand. Blood was collected into a 5 mL bijou tube containing heparin at 7.5 Units per mL blood (Figure 2.3). Group 1 (vaccinated at birth) and Group 2 (vaccinated at 4½ months) had the same venipuncture schedules.

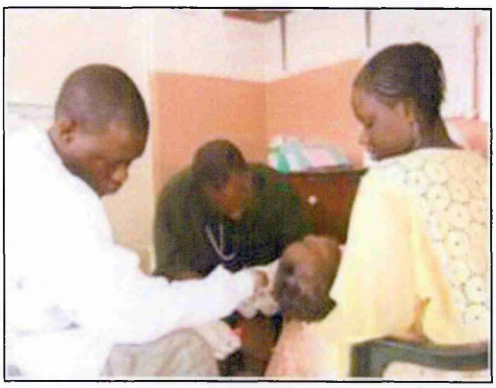


Figure 2.3: Venous blood being collected from a 9 month old child

2.3.3 Urine collection

A urine sample was collected within the first few days of life into a urine bag (Urinocol® Cedex, France) fitted between the baby's legs. The urine was transferred into a 15 mL universal container and used to diagnose CMV infection.

At 4½- and 9- months a urine sample was collected from the infant prior to blood collection and transported to the MRC on the same day. 1 mL of urine was stored at -20°C for later analysis for CMV infection.

2.4 CULTURE CONDITIONS

Whole blood samples were used for all *ex vivo* and *in vitro* work in order to optimise the small volumes of blood collected. To aid in the logistics and practicalities of this study, various protocols were tested including freezing of the blood cells using novel protocols used in field studies in South Africa (Hanekom, Hughes et al. 2004). However FOXP3 expression was found to be very sensitive to freezing methods and therefore fresh blood was used for the entire study. From each blood sample:

1. 100 µL was spun at 10,000 rpm for 5 mins and the plasma layer was aspirated and stored at -20°C
2. 250 µL was used to calculate a full blood count on a CA620-20 Balder parameter system (Boule Medonic, Stockholm, Sweden)
3. 100 µL was used to phenotype the cellular profile of the Tregs *ex vivo* by flow cytometry (600 µl was needed from one donor in each experiment to perform the flow cytometry compensations on the FACSCalibur (BD Biosciences))
4. 500 µL whole blood was cultured for 5 days (optimal time to measure IL-10 and TGFβ production from T cells and maximum time for sufficient cell survival without the aid of growth cytokines) with each of the following

antigens:

a) Purified peptide derivative of tuberculin (PPD, 25μg/mL; SSI, Denmark),
b) Live BCG ($2 - 66 \times 10^4$ CFU/ mL, Serum Institute of India Ltd, India),
c) ESAT-6/CFP-10 fusion protein (EC, 10μg/mL; Klein, M, Leiden),
d) Staphylococcal enterotoxin B (SEB, 5 μg/mL; Sigma Aldrich, UK)
e) Unstimulated control
f) Unstimulated flow cytometry control (after culture combined this control well from each sample for flow cytometry compensations)

Twenty-four hours later the cultures were diluted 1:5 with RPMI medium containing 2% penicillin/streptomycin (10,000 U/mL, Sigma Aldrich, UK) and 1% L-glutamine (Sigma Aldrich, UK). Timing of dilution was optimised to reduce the volume of antigen used without compromising the survival of cells (Figure 2.4).

The superantigen SEB was chosen as the positive control since the cellular flow cytometry profile of the lymphocytes after 5 days of stimulation was similar to the unstimulated whole blood sample and therefore required fewer changes to the analysis of the flow cytometry plots. Any samples that did not respond to SEB were eliminated from the analysis with any of the antigens tested. PPD is a mixture of antigens derived from *M.tb*. Concentrations of PPD and SEB used were optimised from previous infant studies in our laboratory. Responses to PPD could therefore indicate TB disease or latent infection. However it is expected that there is cross reactivity with antigens in the BCG vaccine and NTM. The EC fusion protein consists of two antigens that are relatively *M.tb* specific. It was a kind gift from Dr Michel Klein, Leiden, The Netherlands and used at the recommended concentration from previous ELISpot assays. BCG and most NTM do not contain these antigens although there are exceptions (*M. leprae, M. kansasii, M. marinum, M. smegatus, M. ulcerans*) and therefore some cross reactivity may occur if these NTM are in the environment in The Gambia. The specific environmental mycobacteria common in

The Gambia include *M. intracellulare, M. avium* and *M vaccae* (Corrah 1994). Skin test antigens were used for this study but these are not available for human use at the current time. *In vitro* testing for all these specific antigens was beyond the scope of this study given the small volume of blood available. The BCG antigen was reconstituted in phosphate buffered serum (PBS) as directed for use as a vaccine, from the same vaccine batch as that used for vaccination of the study subjects. The concentration of the BCG was titrated by lymphoproliferation assay. BCG caused inhibition at the higher concentrations. 2 µL per 100 µL cultures was found to be optimal and used for this study. This corresponds to a final concentration of $2 - 66 \times 10^4$ CFU/ mL.

After 5 days of culture with specific antigens:

1. 100 µL of supernatant was collected and stored at -20°C at day 5 of culture to quantify the production of cytokines.
2. 100 µL of cell suspension was collected at the same time point, spun at 10,000 rpm for 5 mins and the pellet resuspended in 350 µl RLT lysis buffer containing RNase inhibitors (QIAGEN, Germany) for RNA extraction and stored at -70°C to study expression profiles at a later date.
3. The remaining cells were divided into three for phenotype intracellular analysis using 3 panels of antibodies (Table 2.2).

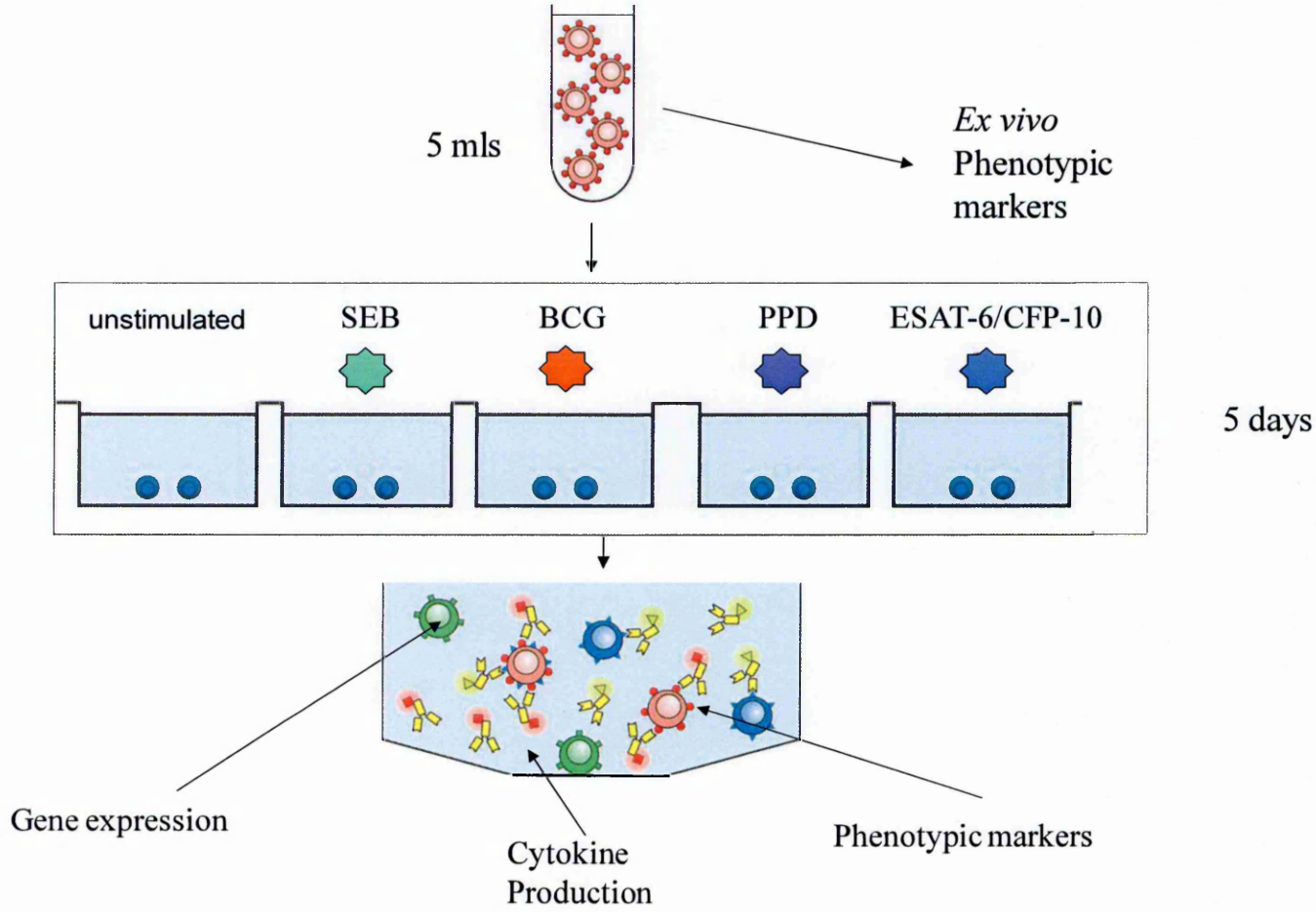


Figure 2.4: Illustration of the laboratory assays (diagrams adapted from (Janeway 2005))

2.5 CELL PHENOTYPING

The selection of cell surface and intracellular antibodies utilised, *ex vivo* and after 5 days of culture are shown in Figure 2.5 and Table 2.2 and the details are listed below:

- BD Pharmingen, Cedex, France
    - CD4 (PE, mouse IgG1 clone SK3), CD4 (APC, mouse IgG1 clone SK3), CD8 (PerCP, mouse IgG1 clone SK1), CD25 (FITC, mouse IgG1 clone M-A251), IL-10 (PE, rat IgG1, clone RM4-5), Ki-67 (FITC, mouse IgG1 clone B56/MOPC-21)
- e-Biosciences, San Diego, US
    - FOXP3 antibody (APC, rat IgG2a clone PCH101)
- IQ Products, Groningen, Netherlands
    - TGFβ (PE, mouse IgG1 clone TB21)

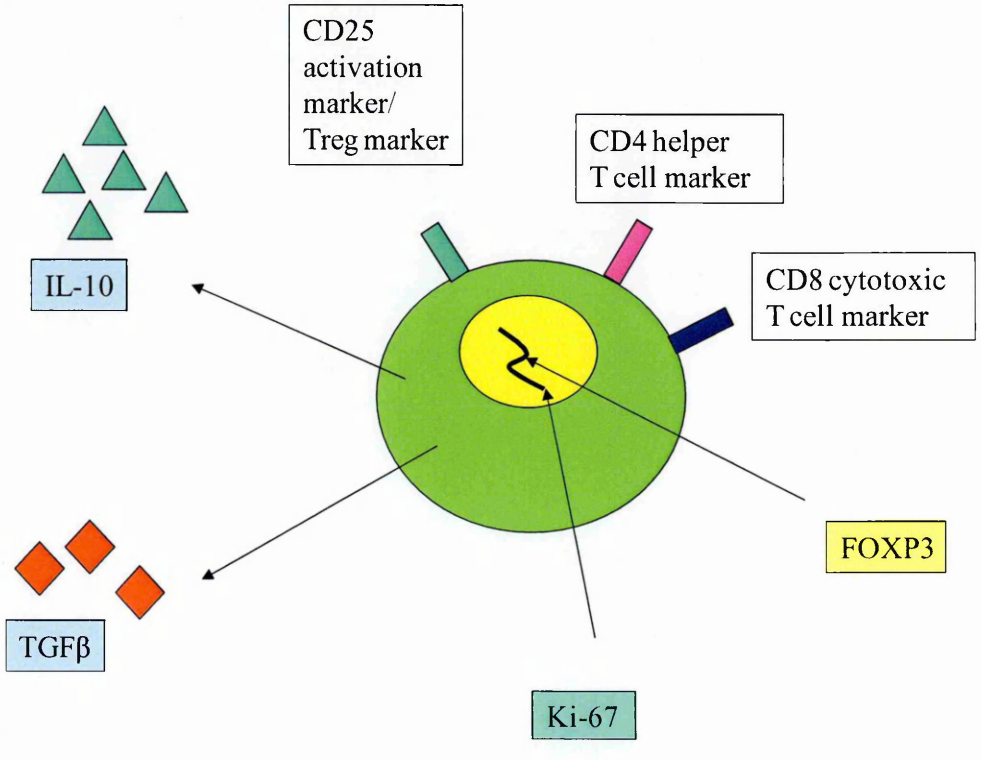


Figure 2.5: Phenotypic markers for flow cytometry analysis

Experiment	FITC (µL)	PE (µL)	PerCP (µL)	APC (µL)
Ex vivo and *in vitro*	CD25 (2)	CD4 (2.5)	CD8 (5)	*FOXP3 (5)
In vitro	*Ki-67 (5)	*IL-10 (10)	CD8 (5)	CD4 (3)
In vitro	CD3 (3)	*TGFβ (5)	CD8 (5)	CD4 (3)

FITC – fluorescein isothiocyanate, PE – phycoerythrin, PerCP - peridinin chlorophyll protein, APC – allophycocyanin
* intracellular staining
( ) denote antibody volumes used per 100 µL

Table 2.2: Flow cytometry antibody panels

Antibody titrations and isotype controls were assessed in cord blood to optimise the flow cytometry staining methods after 5 days of culture.

In humans, the average peak of expression of the activation marker, CD25 (IL-2R) after stimulation with SEB is 6 days with 80% lymphocytes expressing the marker (Caruso, Licenziati et al. 1997) and therefore the percentage of $CD4^+$ T cells that expressed CD25 was chosen to represent activated T cells after 5 days of culture in our study.

There is still no clear definition of naturally occurring Tregs. Many of the markers that are expressed on a suppressive T cell are also expressed during activation of T cells (e.g. HLA-DR, CTLA-4, GITR, LAG-3,). Studies in autoimmunity, reviewed in (Sakaguchi, Sakaguchi et al. 2001) and, more recently, infectious diseases, (reviewed in (Mills 2004)) have hailed FOXP3 as being the most likely candidate to identify Tregs within the $CD4^+CD25^+$ compartment although it remains true that FOXP3 is also transiently upregulated upon activation of human T cells (Morgan, van Bilsen et al. 2005; Allan, Crome et al. 2007). During optimisation experiments upregulation of FOXP3 was found after 1 day of culture which reduced by 5 days, supporting an initial increase in expression upon activation but by 5 days expression of FOXP3 was more likely to be associated with true regulatory T cell activity. Human FOXP3 expression predominates in $CD4^+$ T cells that express high levels of CD25 and it has been suggested that human Tregs

can be defined as $CD4^+CD25^{high}$ T cells (Baecher-Allan, Brown et al. 2003). However this definition remains controversial and is difficult to define by flow cytometry (see Appendix VII for Treg definition optimisations), thus we chose the definition of a Treg as the percentage of $CD4^+$ T cells expressing CD25 and FOXP3. During the study period another marker was identified that has helped define Tregs. $FOXP3^+$ T cells that downregulate the IL-7 receptor (CD127) show highly suppressive properties (Liu, Putnam et al. 2006) and thus $CD127^{low}$ has been accepted, in combination with FOXP3, as a marker of Tregs. It was not possible to include this antibody to our panel for this study but work in our laboratory has shown that 94% of *ex vivo* $CD4^+CD25^+FOXP3^+$ T cells in cord blood have downregulated expression of CD127 (Flanagan, K, personal communication). It is interesting to note that higher levels of CD127 are expressed on cord blood $CD4^+$ and $CD8^+$ T cells, compared to adults and this may falsely reduce the number of Tregs identified using this method in cord blood (Hassan and Reen 2001; Schonland, Zimmer et al. 2003). When other intracellular markers were tested using the recommended FOXP3 intra-nuclear staining protocol there was a high background response, therefore intracellular IL-10, Ki-67 and TGFβ were assessed using separate intracellular staining methods.

IL-10 and TGFβ are associated with inducible Tregs and levels peaked after 5 days of culture, thus determining an optimal culture time.

As the volume of blood was limited, Ki-67, an intracellular marker of cell division, was used as a functional proliferation read out rather than performing separate lymphocyte proliferation assays by thymidine incorporation, bromodeoxyurindine (BrdU) or carboxyfluorescin diacetate succinimidyl ester (CFSE) staining.

Consistency within the analysis of the flow cytometry data was gained by utilising a predefined template for the gating strategies on FlowJo (Treestar Inc, Oregon, US) and not deviating from this for all samples and all time points beyond that needed for subtle variations in sample characteristics. $CD4^+$ and $CD8^+$ antibodies were used in all three

panels for flow cytometry and therefore the consistency of the percentages of cells with these markers indicated the level of inter-assay variability. The percentage of $CD4^+$ and $CD8^+$ T cells within the lymphocyte population was found to be highly consistent across all panels at all time points (data not shown). With respect to the $CD4^+$ population, different $CD4^+$ antibody clones and different fluorochromes were used within the FOXP3 and the IL-10/TGFβ panels. A comparison of $CD4^+$ T cell values between panels was also consistent suggesting inter-assay variability was minimal (data not shown).

2.5.1 Cell surface staining

Red blood cells in whole blood were lysed 1:10 with 1 x red blood cell lysing buffer (diluted from 10 x buffer in PBS, BD Biosciences) for 7 mins. The *in vitro* samples were first collected from the wells of the tissue culture plate and spun at 2000 rpm for 10 mins to pellet the cells before adding the lysis buffer. After 7 mins PBS was added up to 15 mL to dilute the lysis buffer and avoid damage to the cells. The cells were spun at 2000 rpm for 10 mins then washed (spun at 2000 rpm for 5 mins) with FACS buffer (0.5% BSA, 0.1% EDTA, 0.1% sodium azide in PBS) before being resuspended into 100 µL FACS buffer and stained with the cell surface antibodies shown in Table 2.2 for 30 mins at 4°C in the dark. The cells were then washed twice in FACS buffer before permeabilisation for intracellular staining.

For each experiment, unstimulated control cells for the compensation stains were pooled and processed as above before being separated into 5 tubes (100 µL in each tube) as below:

1. Unstained (to set up the voltage parameters)
2. CD3 (FITC, mouse IgG1 clone UCHT1, BD Pharmingen)
3. CD4 (PE, mouse IgG1 cloneSK3, BD Pharmingen)
4. CD3 (PerCP, mouse IgG1 clone SK7, BD Pharmingen)
5. CD3 (APC, mouse IgG1 clone UCHT1, BD Pharmingen)

Each antibody used was previously optimised using isotype controls to assess background staining. Electronic compensation was required for every experiment to minimise spectral overlap detected by the photomultipliers of the FACSCalibur which is dependent on the sample and assay conditions.

2.5.2 Intracellular staining (ICS)

FOXP3 protocol

200 µL FOXP3 permeabilisation reagent (made on same day 1:3 with diluent provided) (e-Bioscience) was added and incubated at 4°C for 20 mins in the dark. The cells were then washed once in FACS buffer and then twice in 1 x FOXP3 permeabilisation buffer (diluted from 10 x stock in distilled water, e-Bioscience). 10% rat serum (e-Bioscience) was added to the cells for 15 mins at 4°C in the dark to block any non-specific binding of the FOXP3 antibody before 5 µL of FOXP3-APC was added for 30 mins at 4°C in the dark. The cells were then washed three times with FOXP3 permeabilisation buffer before resuspending in 150 µL fix buffer (1% formalin in PBS).

BD protocol

200 µL Cytofix/cytoperm (BD Bioscience, France) was added and incubated at 4°C for 20 mins in the dark. The cells were then washed in 1 x BD Permeabilisation buffer (diluted from 10 x stock in distilled water; BD Bioscience) and then incubated in the same buffer for 10 mins at 4°C in the dark. 10 µL of IL-10 and 5 µL of Ki-67 antibodies were added and incubated for 30 mins at 4°C. TGFβ was added after 10 mins for a total incubation of 20 min for this antibody. The cells were then washed three times with BD permeabilisation buffer before resuspending in 150 µL fix buffer (1% formalin).

When there were large numbers of samples, 96 well plates were used for staining

and were transferred to 5 mL round bottom tubes (BD Falcon, Cedex, France) prior to running on the FACSCalibur (BD Biosciences). The maximum numbers of events collected from each sample were equivalent to 5,000 – 50,000 lymphocytes depending on the age of the subject and the culture conditions.

2.6 CYTOMETRIC BEAD ARRAY

Supernatants collected at day 5 were thawed and spun at 1500 rpm for 2 mins to pellet any debris and avoid needle blockage of the Bio-Plex 200 Suspension Array system (Bio-Rad, Hercules, California, US). 50 µL of selected samples were transferred to a 96 well plate in preparation for the assay. All samples stimulated with SEB were diluted 1:2 with media containing 10% serum (simulating the whole blood culture milieu) to detect cytokines within the range of the standard curve.

A 3-plex system to analyse IFNγ, IL-10 and IL-13 cytokine production (Th1 cytokine kit, Bio-Rad) was used to analyse samples collected at birth and at 9 months, and a 6-plex system containing antibodies to IFNγ, IL-10, IL-13, IL-6, IL-7 and IL-17 (Th1 cytokine kit, Bio-Rad) was used to determine cytokine production at 4½ months of age.

Briefly, the lyophilised standard was reconstituted with 500 µL RPMI media containing 10% human serum, gently vortexed and incubated on ice for 30 mins. Serial dilutions of 1:4 were made according to manufacturer's instructions with above media to create 8 standards for the low PMT setting which allows for a broad range standard curve. During all steps of the assay, the reagent solutions in the 96 well plate were removed by vacuum filtration using Aurum™ Vacuum manifold with 1-2 Hg pressure (Bio-Rad), and excess liquid was blotted with a lint free paper towel. The filter plate (Bio-Plex reagent kit, Bio-Rad) was prewetted with 100 µL assay buffer (Bio-Plex reagent kit, Bio-Rad). The conjugated microsphere beads were vigorously vortexed for 20 - 30 secs before being diluted with assay buffer (as per manufacturer's instructions), and 50 µL was added to each well of the plate. The buffer was removed by vacuum filtration. The plate was then washed

twice with 100 µL wash buffer (Bio-Plex reagent kit, Bio-Rad) after which 50 µl standards in duplicate and 50 µL single samples in were added to the plate. The plate was then sealed with sealing tape and incubated by shaking at 1,100 rpm for 30 secs followed by 300 rpm for 1 hour at room temperature in the dark. During the incubation the biotinylated detection antibody (Bio-Plex reagent kit, Bio-Rad) was diluted as per manufacturer's instructions and kept in the dark at room temperature until use. The plate was washed three times with wash buffer as described previously and 25 µL of detection antibody was vortexed gently and added to each well, the plate was sealed with sealing tape and incubated by shaking at 1,100rpm for 30 secs followed by 300rpm for 30 mins in the dark. The streptavidin-PE reporter antibody (Bio-Plex reagent kit, Bio-Rad) was diluted as per manufacturer's instructions, vortexed rigorously and after another 3 washes with wash buffer, 50 µL was added to each well of the plate and the plate was sealed and incubated for 10 mins (shaking at 1,100rpm for 30 secs followed by 300 rpm). The plate was finally washed three times with wash buffer and then each well was resuspended with 125 µl of assay buffer, shaken for 30 secs at 1,100rpm before reading the plate on the Bio-Plex 200 Suspension Array system (Bio-Rad).

Standard curve outliers were eliminated by identifying samples where the coefficient of variance (%CV) > 10% and observed/expected x 100 (obs/exp*100) was outside the range of 100 +/- 20. Any sample that recorded an error during the run (predominantly due to high aggregations of beads) was repeated. Calibration of the instrument was performed before every experiment using CAL2 calibration kit (Bio-Rad) with low RP1 target values (used for broad range standard curve).

2.7 CMV DIAGNOSIS

2.7.1 CMV DNA extraction

CMV viral DNA was extracted from 140 µL urine using the QIAmp viral RNA kit (QIAGEN, Hilden, Germany) as recommended by QIAGEN for nucleic acid extractions

from non-cellular material. Urine from a known CMV infected subject was used as a positive control and PBS was used as a negative control.

Carrier RNA was utilised to increase the yield from the silica columns. It was prepared fresh, reconstituting in AVE buffer and diluting in AVL buffer as per manufacturer's instructions. 560 µL of prepared RNA was added to 1.5 mL tubes (this equated to 5.6 µg yeast tRNA per sample). 140 µL sample urine was added to the carrier RNA and vortexed for 15 secs and incubated at room temperature for 10 mins. 560 µL of 100% ethanol was added to each tube, vortexed for 15 secs and 630 µL of the sample was applied to a QIAamp Mini spin column before being spun at 8,000 rpm for 1 min. This was repeated to bind the entire DNA to the silica columns. The spin columns were washed with 500 µL buffer AWI at 8,000 rpm for 1 min, then secondly with 500 µL buffer AW2 at 14,000 rpm for 3 mins. As an extra step, the column was spun at 14,000 rpm for 1 min without any buffer to remove any residual alcohol. The DNA was then eluted in two steps using 40 µL of AVE buffer for each step and stored at –20°C.

Measurement of the yield of CMV DNA would be confounded by the carrier RNA eluted in the process, but purity of the sample was calculated on the Nanodrop (Thermo Scientific, Wilmington, Delaware, US) based on absorption values at 280 nm (protein), 230 nm (detection of contaminating organics/ proteins), 270 nm (detection of phenol contaminates) using the following criteria: 260 nm / 280 nm >1.8, 260nm / 230 nm >1.8 and 260 nm / 270 nm ~1.2. If any sample was outside these limitations then the extraction was repeated.

2.7.2 CMV nested PCR

In the first round of PCR, 10 µL DNA was amplified using Hot Star *Plus* Taq polymerase kit (QIAGEN) following manufacturer's instructions in a 25 µl total volume and with 0.1 µM CMV primers (WP1 forward – 5' GTA CTC GCC CTC GTT TTC GGG TC 3' and WP2 reverse – 5' GTC TAT TGT GTC GAG TAT CTA CTC AG 3'). These

primers amplified part of the membrane-associated protein UL50 gene of the human herpesvirus 5 (laboratory strain AD169) which is highly homologous to the wild type human herpesvirus 5 Merlin strain. The CMV strain from The Gambia had not been sequenced but a BLASTn comparison of the primer sequences to genomic DNA from the NCBI database elicited a 100% homology to the laboratory strain and 98% homology to the wild type strain suggesting that it was likely the primers would amplify the Gambian strain. Extracted controls, mentioned above and a PCR negative control (10 µL of nuclease free water instead of sample) were amplified alongside the samples. PCR conditions were:

- 94°C, 4 mins – 1 cycle
- 94°C, 30 secs, 50°C 30 secs, 72°C 30 secs – 4 cycles
- 94°C, 30 secs, 57°C 30 secs, 72°C 30 secs – 31 cycles
- 72°C, 7 mins – 1 cycle

In the second round of PCR, 2 µL first round DNA product was amplified using Hot Star *Plus* PCR kit (QIAGEN) following manufacturers' instructions in a 20 µl total volume and using 0.1 µM CMV primers (WP3 forward – 5' TCC GAA GGC GAT GAG CTC GAT GT 3' and WP4 reverse – 5' CGA GTA TCT ACT CAG CTA CTG GGA 3') that amplified within the first PCR product. PCR amplification conditions were same as above for the first round PCR.

10 µL second round PCR product was added to 2 µL of 6 x loading buffer (Promega, Madison, US) and ran on a 2% agarose gel (low melting point agarose, Sigma Aldrich, Poole, UK) in 1 x TAE buffer (50 x stock – 242 g Tris, 100 mL 0.5M EDTA, pH 8.0, 57.1 mL glacial acetic acid made up to 1 L with water) at 100 volts alongside 1 kb molecular weight marker (Promega, Madison, US).

2.8 DATA COLLECTION AND VERIFICATION

Anthropometric information (at birth and at monthly follow ups), TB questionnaire,

case report forms (CRF) forms, vaccine records, drop out forms, full blood count results and storage location for samples were all double entered onto a central Access database and original forms filed in one locked location. Results from flow cytometry, CBA and PCR were recorded on Excel databases.

Flow cytometric data was analysed using a template previously created during optimisations in Flow Jo software (Treestar, Oregon, US) with minor sample specific modifications. Cytokine concentrations below the level of detection (indicated as OOR in Bioplex software) were calculated as zero in the analysis. All values that were greater than the highest range within the standard curve were repeated by first diluting 1:2 with media.

Data points/samples were eliminated if any of the following problems arose:

1. EPI vaccine less than 7 days prior to blood collection
2. Cord blood was clotted
3. Cultures were contaminated
4. Flow cytometry antibodies did not work optimally
5. The positive control (SEB) did not induce a response *in vitro*
6. Samples with acquisition of less than 1,000 cells (in the case of an unstimulated well having less than 1,000 cells, all antigen stimulations from the same sample were removed from the analysis)
7. Technical problems with the FACSCalibur causing problems with acquisition of data

Outliers were always included in the data analysis unless mentioned otherwise.

2.9 STATISTICAL ANALYSIS

A test of Gaussian distribution was initially performed using the D'Agostino-Pearson normality test (omnibus K2). For most values the data was not normally distributed and therefore non-parametric testing was used throughout.

Cross sectional comparisons between groups at each time point were assessed using

a non-parametric 2-sided Mann-Whitney U test at 95% significance that tests differences in median values but also the distribution of the data (location and shape). This test is described with respect to medical literature by Hart, 2001 in a revision of analysis from BMJ articles (Hart 2001). All paired analysis (i.e. unstimulated compared to stimulated samples or longitudinal data) was analysed using non-parametric 2-sided Wilcoxon matched paired test at a 95% significance level which analyses related samples by comparing the ranks of the differences, regardless of the sign of the difference and ignoring all zero differences.

Correlations were analysed using Spearmans non-parametric correlation coefficient as opposed to linear regression as X and Y are both variables that were measured.

The categorical data assessing gender differences were recorded as contingency tables and analysed using Fisher's Exact test for small sample sizes.

Bonferroni corrections were used according to the numbers of parameters assessed and the number of times a dataset was analysed. Corrected and uncorrected data were reported.

CHAPTER 3

Cohort Characteristics

3.1 THE GAMBIA

The Gambia is the smallest country in Africa situated at the furthest Western point of the continent surrounded by Senegal (Figure 3.1).

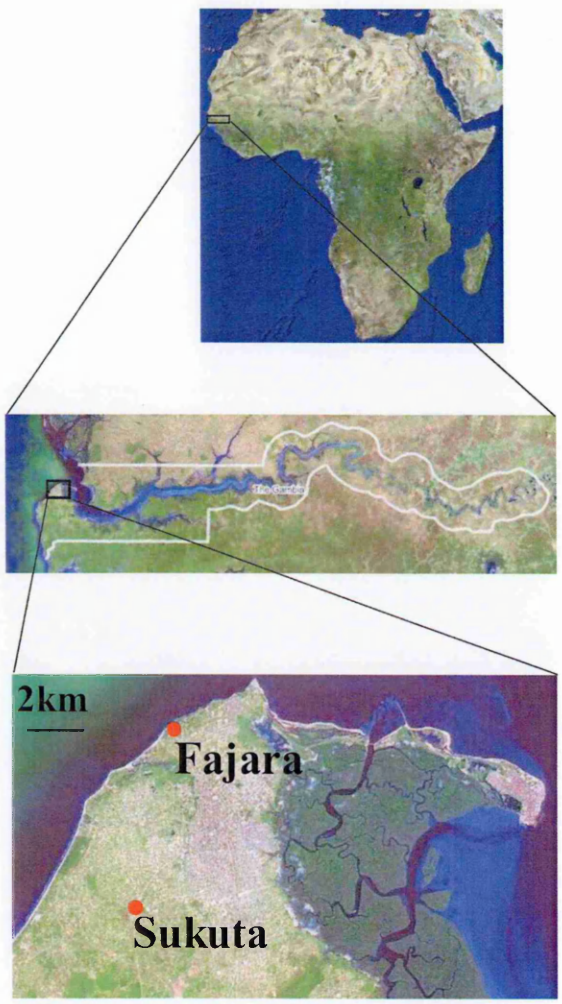


Figure 3.1: Map of Gambia and location of Sukuta field site

The country is approximately 300 km long and 40 km wide, with approximately 3,700 km of roads (80% unpaved, (CIA 2008)) and a population of 1.74 million (CIA 2008). The River Gambia, the origin of which lies in Guinea Conakry, runs from East to West through the entire country and is 8 km wide at its widest point. At 13° latitude, The Gambia experiences a tropical climate that consists of a wet season (June – October) and a dry season (November – May). The Gambia has no confirmed mineral resource deposits

and has a limited agricultural base although 75% of the population depends on crops and livestock for its livelihood (CIA 2008). Although Gambia has a history of many successful small scale industries including ground nut export and fishing, its most lucrative industry is tourism, with approximately 200,000 tourists, mainly from Europe, visiting Gambia every year to experience Gambia's natural beauty, tropical birds, sandy beaches and winter sun. The gross domestic product per capita (GDP PPP) in 2008 was estimated to be $1,200 compared to the UK estimation in 2008 of $37,400 (CIA 2008). The majority religion is Islam (90%) while the remaining includes Christians (8%) and those of indigenous beliefs (CIA 2008).

3.2 CHILD HEALTH IN THE GAMBIA

Reducing child mortality by two-thirds is one of the Millennium Goals to be achieved by 2015. Globally, about 29,000 children under the age of 5 die every day (21 every minute) from preventable causes (UNICEF 2009). Life expectancy in The Gambia is 54.95 years of age (male 53.06 and female 56.9 years) (UNICEF 2006). The birth rate is one of the highest in the world at 38.36 births per 1,000 population (CIA 2008) but there is a high mortality rate, especially in under 5 year olds (113 per 1,000 children) (UNICEF 2006). However, The Gambia has been one of the fastest improvers in under-five mortality rates among developing countries (UNICEF 2006). A study in the rural upper river regions showed acute lower respiratory tract infections (ALRTI) were the most frequent cause of death in children under 5 years of age. Other major causes were malaria, acute gastro-enteritis and chronic diarrhoea with malnutrition. During the neonatal period (< 28 days of life) children were more susceptible to ALRTIs, whereas malaria and chronic diarrhoea were more frequent causes of mortality from 1 – 5 years of age (De Francisco, Hall et al. 1993). From 1949 to 1997 the probability of dying in The Gambia was highest for 1- 5 year old children (66 per 1,000) compared to neonates (15 per 1,000) and infants (1 month to 1 year) (36 per 1,000) (Rayco-Solon, Moore et al. 2004). Mortality used to be greatest in

the 'hungry' season (July – November), although this seasonality of deaths was lost from 1985 onwards (Rayco-Solon, Moore et al. 2004). This is thought to be due to the more efficient implementation and availability of basic health measures. The neonatal mortality rate of 15 per 1,000 was low compared to earlier reports of 39 per 1,000 (Leach, McArdle et al. 1999) and the UNICEF figure of 46 per 1,000 (UNICEF 2000). Leach *et al* showed that infections were responsible for 57% of the neonatal deaths (Leach, McArdle et al. 1999). Infectious diseases have been reduced by the implementation of the EPI in 1979 which has been very successful with up to 90% of infants throughout the country being vaccinated with the recommended vaccines (see Table 2.1)(WHO 2008). Receiving all recommended vaccinations before the age of 9 months has had a positive effect on preventing under-5 child mortality in The Gambia (Rutherford, Dockerty et al. 2009).

It is estimated that the incidence of TB disease in The Gambia is 603 per 100,000 (Hill, Jackson-Sillah et al. 2008). A recent surveillance of TB incidence from the greater Banjul area of The Gambia revealed Sukuta as a 'hot spot' for TB cases. However 80% of the detected cases occurred in the 15 – 49 year age group and only 1.6% in < 1 year olds which is the age of the children in our study (Touray, K personal communication).

3.3 SUKUTA FIELD SITE

Sukuta is a semi-rural community about 15 minutes drive from the main MRC field station at Fajara (Figure 3.1). The field site is based at Sukuta Government Hospital run by a prominent local figure called 'Aunt Sally', a senior nurse who is dedicated to the running of the hospital.

Figure 3.2: Sukuta field team and infant immunology lab staff. From left to right; back row *- Momodou Cox, Prof. Hilton Whittle, Momodou Bah, Saidou Sowe, Ebrima Touray,* ***second row*** *- Suleyman Colley, Buba Darbo, Public Health Officer at Sukuta Health Centre, Janco Camara, Saiho Bob, Dr. Owolabi Olumuyiwa, Sargo Sanneh, Karamou Manneh, Omar Badjie,* ***third row*** *- Fatou Noho Konteh, Jankey Ya Jagne, Isatou Drammeh, Lady Chilel Sanyang, Aunty Sally, Dr. Jane Adetifa, Musa Sambou, Saikou Mendy, Jammo Sowe,* ***bottom row crouching*** *- Jainaba Njie-Jobe,* ***Sarah Burl****, Dr. Katie Flanagan, Ebu Bah.*

Within the compound of the clinic is a smaller clinic dedicated to MRC studies, which was established by Prof. Hilton Whittle, Dr. Arnaud Marchant and Dr. Marianne van der Sande in 2001 to study CMV infection. Since this time many studies have been carried out and a birth cohort has been established. The site is now used to study infections that affect infants in early life and to investigate responses to infant vaccines (current and novel). There is one MRC clinician, Dr. Jane Adetifa and 13 MRC field workers, 5 with nursing qualifications (Figure 3.2) based at the field site. Each fieldworker has a motorcycle to travel within the 40 km catchment area to visit study participants throughout

the dry and wet seasons.

3.4 STUDY SET UP

According to International Conference of Harmonisation/ WHO Good Laboratory Practice (ICH-GCP) guidelines (www.ich.org) any clinical intervention study in human subjects must be set up as a clinical study. Although BCG is an already licensed vaccine, changing the usual schedule could introduce risk to a human subject and therefore it was thought advisable to set up the study using the MRC GCP guidelines that are based on the principles of the ICH GCP guidelines. After 50 subjects had been recruited the study was monitored internally by the clinical trial monitor with no major underlying problems.

3.5 STUDY DROP OUT ANALYSIS

103 babies were recruited into the study at birth. The final dropout rate was 15.6% which was below the original estimated rate of 20%. Of the 103 subjects recruited, 91 were followed up at 4½ months (88.3%) and 87 by 9 months (84.4%). Table 3.1 illustrates the details of the 'drop outs' of the study.

REASONS FOR DROP OUT	0 - 4½ m	4½ - 9 m	at 9 m
Died	3	0	0
TB exposed	1	0	0
TB disease	0	0	0
Withdrew consent	2	0	1
Travelled	0	1	2
Protocol deviation	6	0	0
TOTAL	**12**	**1**	**3**

Table 3.1: Reasons study participants dropped out of study over the 9 months of the study

Three babies died within the first month of life (2/3 were within 2 days of birth).

One of these was diagnosed as having sepsis but the remaining two had general fever and breathing difficulties that were undiagnosed before death. This would approximate to a neonatal mortality rate (NMR) of 30 per 1,000 live births (official Gambia NMR is 46 per 1,000 live births (UNICEF 2000)). In 2006, the NMR of England, Wales and Northern Ireland was 3.4 per 1,000 live births (CEMACH 2006). Thus, this study suggests a 10-fold higher NMR in Sukuta than in a Western setting, although the sample size was too small to extrapolate this to a nationwide statistic and there was a selection bias of recruitment birth weight > 2.5kg (in 2007, 6% of births in Sukuta were < 2.5kg).

3.6 DOCUMENTATION

The CRFs and source documents used in this study are summarised in Table 3.2. An access database was created to hold the data generated by the 2731 forms completed.

Forms entered into database	Number collected
Recruitment form at birth	103
First visit form	95
Monthly follow up form	726
TB questionnaire	1101
Infant Welfare Card (IWC) vaccine details	103
Case Report Forms (CRF) for bleeding and TST	177
Drop out forms	16
Results from Medonic cell analyser	101
Storage location forms for all laboratory samples	309
TOTAL	**2731**

Table 3.2: Number of forms collected for the duration of the study

A recruitment form for each subject recorded details of the parents and birth of the child. A first visit form is filled out to record the child's given name and details within the first month of life (traditionally children in The Gambia are given a name by the father of

the child at a naming ceremony which is usually within the first 2 weeks of life). From month 2 until month 9 each child was followed up on a monthly basis and a general health form and a TB exposure questionnaire were completed. On average, >75% of monthly follow up forms were completed, the absent forms being mainly due to the family travelling at time required for follow up. Each child that dropped out of the study was recorded, with detailed reasons as listed above in Table 3.1. A CRF was recorded for each blood collection and TST administered, along with details of the personnel involved with collection of blood or measurement of the TST.

3.7 VACCINE SCHEDULE

Each mother of the study participants was advised to bring their child to the MRC field site at Sukuta for their child's EPI vaccines so that a record could be maintained of each vaccine administered. This information was recorded on the child's Infant Welfare Card (IWC; a form that is given to every newly born child in The Gambia and records vaccines administered in addition to height and weight). MRC field staff recorded the vaccines given, on a database and a schedule of follow up was set up to maintain the appropriate vaccine schedules for each child. Although this intense follow up was implemented there were still cases of blood collection at 4½- or 9- months of age that occurred less than 1 week post vaccine administration. It is not known what effect vaccines have on *in vitro* responses to other antigens, thus these individuals were removed from the final analysis.

3.8 OVERALL COHORT CHARACTERISTICS

Fifty males and 53 female newborn babies were recruited into the study. In Group 1 27/53 (50.9%) were female and in Group 2 26/50 (52.0%) were female suggesting no sex bias in the randomisations of the groups. All babies were cephalic spontaneous vaginal deliveries without complications. According to the criteria of the study the weight of the

child recruited at birth had to be at least 2.5 kg. Two babies with a birth weight below 2.5 kg were mistakenly recruited at birth but immediately dropped out as indicated as protocol deviation in Table 3.1, and these values were eliminated from the length and weight values presented in Table 3.3.

Characteristic		
Gender (%)	Male 50	Female 53
Ethnicity (%) • Mandinka • Fula • Wolof • Other	Mother 67 10 8 15	Father 64 18 7 11
No. of Pregnancies (range) • Total including miscarriages • Live deliveries	 3.8 (1 – 10) 3.6 (1 – 9)	
Mother's median age (range)	26 (18 – 45)	
Lived in Sukuta (%) • < 2 yrs • 2 - 10 yrs • > 10 years • always	 6 (6.0%) 31 (30.3%) 23 (22.5%) 42 (41.2%)	
Birth weight (average kg)	3.2 (2.5 – 4.2)	
Birth length (average cm)	49.5 (46 – 52.1)	

Table 3.3: Cohort characteristics

Sixty five percent of mothers had lived in the community for greater than 10 years with nearly half of all mothers being permanent residents for their entire life (41.2%)(Table 3.3). This suggests the study participants were from a stable population within Sukuta and would have similar social pressures over the time of the study. Mandinka was the prominent ethnic group with 67% of mothers and 64% of fathers from this group. The second most prominent group was Fula and Wolof was third with only 8% of mothers and 7% of fathers from this group (Table 3.3). This proportion is more consistent with rural

areas of The Gambia rather than the urban areas of Banjul and Fajara (where MRC's main unit is based) that are predominantly Wolof speaking. Sukuta is therefore termed semi-rural (or peri-urban) as it is close to an urban area but has a setting and community more usually seen in rural areas. These statistics are similar to previous larger studies in Sukuta (Miles, Sande et al. 2008).

All mothers breastfed their children for the duration of the study (9 months) with daily supplements of water, porridge, rice, coos (couscous) or custard starting from approximately 3 months. The majority of children were taking some solid supplements, by 9 months of life.

The median number of pregnancies, including miscarriages of the mothers of the study subjects was 3.8 with a range of 1 to 10 but numbers of live births was 3.6 with a range of 1 to 9. The median age of the mothers was 26, which is equivalent to the overall birth cohort in Sukuta which has a median age of 25 (n = 703) (Hossin, S personal communication).

Overall it was found that the characteristics of this study cohort were similar to previous studies in Sukuta and therefore suggest this group of children are representative of the Sukuta community.

CHAPTER 4

Ex vivo immune cell populations and phenotypes

4.1 INTRODUCTION

Studying the development of infant immunity in humans is difficult for experimental, practical and ethical reasons and therefore many studies are based on more easily available cord blood or detailed newborn mouse studies. Immune cells in cord blood, however, are predominantly naïve and have different characteristics and dynamics than later in life (Keever 1993; Ciccimarra 1994; Roncarolo, Bigler et al. 1994; Zola, Fusco et al. 1995; Fadel and Sarzotti 2000; Szabolcs, Park et al. 2003) and therefore there is a need to study responses after birth in order to understand how the infant immune system matures particularly in response to pathogens and vaccines encountered in early life. Additional factors such as geographical location (Das, Bhanushali et al. 2008) and genetic background (Newport, Goetghebuer et al. 2004) also influence immune development in early life.

Antibody mediated immune responses are supplemented by circulating transplacentally acquired maternal antibodies which wane by 6-9 months of age, in addition to antibodies received through breast feeding, and these aid in protecting the infant from infections to which the mother has developed immunity. There is limited evidence of the contribution of maternal memory T cells to newborn immunity, and therefore they remain susceptible to those infections that are cleared by a cell mediated response, such as intracellular infections.

Immune cell populations in whole blood can be determined using a cell analyser. Normal ranges have been established as 'reference ranges' and deviation outside of these limits may indicate disease (Table 4.1).

	Concentration in blood (x 10^9 cells /L)		Percentage of blood (%)	Size (μm)
Erythrocytes	*Male*	*Female*	~50	6 - 8
	4400 – 5900	3800 – 5200		
Platelets	150 - 440		ND	Irregular
White blood cells (leukocytes)	4 - 11		~50	7 - 20
• **Granulocytes**			**% of WBC**	
○ **Basophils**	0 - 0.2		0 - 1	9 – 12
○ **Eosinophils**	0 - 0.45		1 - 3	10 – 14
○ **Neutrophils**	2.1 - 7.2		55 - 65	9 – 12
• **Lymphocytes**	1.5 - 4.0		20 - 40	7 – 20
• **Monocytes**	0.2 - 0.8		4 - 10	15 – 20
• **NK cells**	0.004 – 0.02		1 - 5	7 - 20

ND = not determined

Table 4.1: Normal composition of peripheral blood in adult Caucasians

Various studies have shown that age (Lugada, Mermin et al. 2004; Adetifa, Hill et al. 2008; Sirdah, Tarazi et al. 2008), sex (Bain 1996; Jackson, Carter et al. 2001; Rushton, Dover et al. 2001; Lugada, Mermin et al. 2004; Adetifa, Hill et al. 2008) and ethnic origin (Alur, Devapatla et al. 2000; Cheng, Chan et al. 2004; Lugada, Mermin et al. 2004; Robins and Blum 2007; Adetifa, Hill et al. 2008; Saathoff, Schneider et al. 2008; Sanz-Pelaez, Angel-Moreno et al. 2008; Sirdah, Tarazi et al. 2008) can lead to a change in the expected normal range. There are little data on what is an appropriate normal range for an African infant population, but studies based in various African settings have shown increased haemoglobin (Hb), packed cell volume (PCV), mean cell haemoglobin (MCH) and numbers of red blood cells (RBC) in cord blood from normal healthy neonates (Mukiibi, Mtimavalye et al. 1995; Mukiibi, Nkrumah et al. 1995; Dapper and Didia 2006; Christensen, Jopling et al. 2008). Sex differences are thought to only appear after puberty, where females have lower haemoglobin and red blood cell counts but higher neutrophil counts (Bain 1996; Jackson, Carter et al. 2001; Rushton, Dover et al. 2001; Lugada, Mermin et al. 2004; Adetifa, Hill et al. 2008). Sex differences prior to puberty have not

been well described.

Paediatric studies of lymphocyte subsets have described differences according to age, many of these occurred over the first year of life (Shearer, Rosenblatt et al. 2003). Changes in *ex vivo* (straight from collection, prior to *in vitro* stimulation) lymphocyte subsets can occur in response to infections (chronic or acute), stress, hormonal changes and even circadian rhythms (Laurence 1993). Very few studies describe the phenotype of circulating T cells in infants related to vaccination but rather focus on *in vitro* antigen specific responses. It might be expected that immediately after vaccination there would be a difference in the peripheral lymphocyte subsets as documented by Eibl *et al* after tetanus toxoid vaccination (Eibl, Mannhalter et al. 1984) but Berrington *et al* did not find this to be the case. Percentages of $CD3^+$ T cells that were $CD4^+$, $CD8^+$ and $CD4^+CD25^+$ at 6 – 8 weeks of age, prior to immunisations were similar to 7 months post infant vaccinations (Berrington, Barge et al. 2005).

Suppressive Tregs in human cord blood have been defined as $CD4^+CD25^+$ or $CD4^+CD25^{high}$ (Ng, Duggan et al. 2001; Wing, Ekmark et al. 2002; Berrington, Barge et al. 2005; Godfrey, Spoden et al. 2005) (Byrne, Stankovic et al. 1994) (Cupedo, Nagasawa et al. 2005). There are conflicting opinions as to whether these Tregs are naïve ($CD45RA^+$) (Takahata, Nomura et al. 2004) or are primed *in utero* and possess a memory phenotype in the cord blood (Paganelli, Cherchi et al. 1994; Brustoski, Moller et al. 2006; Holm, Svensson et al. 2006; Santner-Nanan, Seddiki et al. 2008) (Izcue and Powrie 2005). Wing *et al* describe the phenotype $CD4^+CD25^+$ as being activated/memory T cells and $CD4^+CD25^{++}$ as being Tregs with a predominantly naïve phenotype (Wing, Ekmark et al. 2002). Interestingly, the proportion of these Tregs appeared to decrease during gestation to adult-like levels at birth (Takahata, Nomura et al. 2004; Michaelsson, Mold et al. 2006). More recently the use of CD127 and FOXP3 has further defined cord blood Tregs as $CD4^+CD25^+FOXP3^+CD127^{low}$ (Figueroa-Tentori, Querol et al. 2008) and these constitute

approximately 6% of circulating lymphocytes, the majority having a memory phenotype ($CD45RO^+$) (Santner-Nanan, Seddiki et al. 2008).

This chapter describes the numbers and phenotype of circulating blood cell populations (in particular, Tregs) without stimulation (*ex vivo*) in early life (at birth, 4½- and 9- months of age) and examines if BCG vaccination alters the proportions of these populations.

4.2 RESULTS

4.2.1 Differences in blood cell indices within the first 9 months of life

Whole blood cell counts were measured using a Medonic cell analyser described in Chapter 2: Materials and Methods. The cell counts were not performed for every sample due to the limited quantities of whole blood. Platelets were omitted from the analysis due to increased adhesion and clumping of platelets making the counts unreliable. A larger increase in platelet adherence than normal red cells has been found previously in human newborn cord blood (Aarts, Bolhuis et al. 1983). The results were compared to indices based on children aged between 12 months and 10 years from Kilifi, Kenya (Kenya Medical Research Institute/ Wellcome Trust Research programme), and the Gambia where available (Adetifa, Hill et al. 2008) and cord blood indices from an older Zimbabwean study (Mukiibi, Nkrumah et al. 1995).

It was evident that cord blood immune cells had a different profile to cells during infancy. Cord blood indices were frequently out of the ranges described for children > 12 months of age but were within the range reported from cord blood studies in Zimbabwe (Figure 4.1 and Table 4.2). Cord blood contained significantly higher levels of haemoglobin than infants ($p < 0.0001$). However, using appropriate reference ranges for age, 28% of children were anaemic at birth and 12% of infants were anaemic at both 4½- and 9- months (Figure 4.1A and Table 4.2). The number of red blood cells in cord blood fell within the expected range but were reduced compared to 9 month old infants (Figure 4.1B) and, on average, were greater in mass (PCV/Haematocrit), size (MCV) and haemoglobin concentration (MCHC, MCH) than infants (Table 4.2).

The significantly higher WBC counts in cord blood compared to infants (Figure 4.1C) did not correspond to the proportions of lymphocytes which showed the opposite pattern and were lower in cord blood compared to 4½ months ($p = 0.0010$) and 9 months of age ($p = 0.0003$) (Figure 4.1D).

At 4½ months, The WBC count values appear to define two distinct populations of children with values below the median (although all within expected range) and above the normal range (Figure 4.1C). These two populations were not related to BCG vaccination status. Indeed, when the results were separated according to BCG vaccine schedule, no significant differences were found for any of the indices at any of the time points.

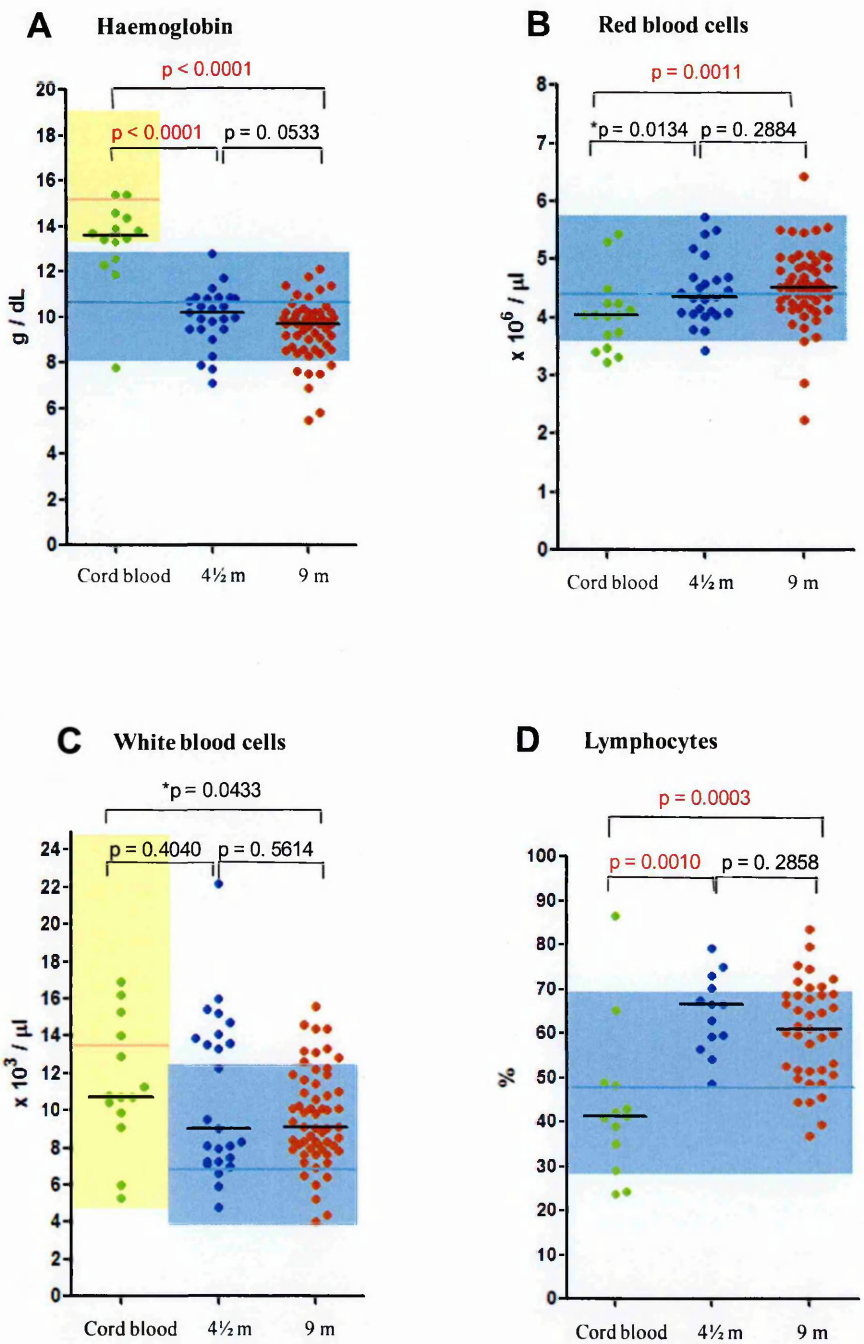


Figure 4.1: Medonic blood counts. *(A) haemoglobin, (B) red blood cells, (C) white blood cells and (D) lymphocytes were determined from 250 µl whole blood using a cell analyser. Cord blood n = 13 - 16, 4½ months n = 13 - 25, 9 months n = 39 - 59. Black bar represents median value and the blue shaded region represents the acceptable range of values from Kilifi, Kenya for children 12 months – 10 years; the median value of the range is represented by a blue line. The yellow shaded region represents the cord blood ranges the pink line represents the median value from Zimbabwe. Mann Whitney U test was applied to compare differences across ages at 5% significance, * represents adjusted values after correcting for multiple testing that were not significant.*

	Cord blood	Reference for cord blood*	4½ months	9 months	Reference (12m – 10yrs)
Haemoglobin (Hb) g /dL	13.6 (7.8 – 15.4)	15.2 (13.5 – 19.4)	10.2 (7.1 – 12.8)	9.7 (5.5 – 27.2)	10.7 (8.2 – 12.7)¶ 8.7 – 13.4ǂ
White blood cell (WBC) x 10^3/µl	10.75 (5.3 – 16.9)	13.8 (4.6 – 132.8)	9.0 (4.8 – 22.2)	9.1 (4.0 – 15.6)	6.9 (3.9 – 12.5)¶ 4.9 – 14.3ǂ
Lymphocytes %	41.3 (23.6 – 86.5)	ND	66.6 (48.6 – 79.2)	64.9 (36.7 – 83.4)	48.3¶ (28.1 – 68.7)
Red blood cell (RBC) x 10^6 /µl	4.03 (3.22 – 5.43)	ND	4.35 (3.43 – 5.73)	4.52 (2.23 – 6.43)	4.4¶ (3.6 – 5.7)
Packed cell volume (PCV) %	40.45 (23.7 – 47.3)	47.3 (38.6 – 60.3)	30.7 (9.5 - 38)	30.30 (17 – 75.4)	32.6¶ (26.8 – 38.1)
Mean cell volume (MCV) fL	101.6 (71.6 – 116.9)	107.8 (88.8 – 134.3)	73.5 (51.4 – 80.7)	67.6 (49.2 – 86.1)	74 (56.3 – 88.7)¶ 63.4 - 86.3ǂ
Mean cell Haemoglobin concentration (MCHC) g /dL	33.55 (32.1 – 34.5)	32.9 (30.3 – 38.3)	31.8 (29.3 - 35)	31.65 (20.3 – 34.8)	32.9¶ (30.0 – 34.9)
Mean cell Haemoglobin (MCH) pg	34.27 (23.6 – 36.4)	31.9 (25.2 – 45.2)	23.80 (16.5 – 27.7)	21.28 (13.6 – 64.2)	24.7¶ (17.0 – 29.7)

* Based on study in Zimbabwe, 1995 (Mukiibi, JM *et al* 1995)
¶ Based on ranges from Kilifi, Kenya (12 months – 10 years, Kenya Medical Research Institute/ Wellcome Trust Research programme)
ǂ Based on Gambian study (Adetifa, IMO *et al*, 2008) (6 months – 6 years)
ND = not determined

Table 4.2: Medonic blood counts. *Median and ranges of all blood indices from 250 µL whole blood analysed by a cell analyser alongside the normal acceptable ranges in Kilifi for children 12 months – 10 years and cord blood ranges from Zimbabwe. Cord blood n = 13 - 16, 4½ months n = 13 - 25, 9 months n = 39 – 59.*

4.2.2 Gender differences in blood cell indices

Analysis by gender revealed no significant differences for any of the indices at birth, but a number of differences emerged at subsequent time points. Many of these differences were lost in the 4½ month age group after correcting for multiple testing, but clear differences persisted at 9 months if age. Females had higher levels of haemoglobin, PCV, MCV and MCH than males (Table 4.3); albeit at still significantly lower levels than in cord blood ($p = 0.0001$). At 4½- and 9- months of age collectively, 80% (8/10) of anaemic infants were male. However at birth this was not the case since 75% (3/4) of anaemic neonates were female (Figure 4.2).

	At birth			4½ months			9 months		
	M	**F**	***p* value**	**M**	**F**	***p* value**	**M**	**F**	***p* value**
Haemoglobin (Hb) g /dL	14.4	13.4	0.3856	9.5	10.5	*0.0115	9.2	9.95	0.0040
White blood cell (WBC) x 10^3 /µl	10.7	10.8	0.4629	8.55	9.5	0.7814	9.0	9.6	0.6075
Lymphocytes %	45.3	40.8	0.4140	72.9	61.1	0.0786	52.25	64	0.720
Red blood cell (RBC) x 10^6 /µl	4.01	4.03	0.7332	4.34	4.42	0.4212	4.54	4.435	0.3060
Packed cell volume (PCV) %	43.2	40.2	0.4278	29.45	33.35	*0.015	29.1	31.80	*0.0065
Mean cell volume (MCV) fL	89.3	102	0.2573	64.75	75	*0.0051	64.1	70.15	< 0.0001
Mean cell Haemoglobin concentration (MCHC) g /dL	33.2	33.60	0.4222	31.4	32.2	0.2117	31.95	31.45	0.9442
Mean cell Haemoglobin (MCH) pg	29.06	34.3	0.6064	20.54	24.44	*0.0158	20.21	22.28	0.0005

*Not significant after Bonferroni correction, blue text denotes greater values in females

Table 4.3: Gender differences in blood cell counts. *Median and ranges of all blood indices from 250 µL whole blood analysed by a cell analyser and divided into groups according to gender. Differences between sexes were calculated using Mann Whitney non-parametric test at 5% significance. * represents values that were not significant after*

adjustment for multiple testing. Cord blood; male n = 5, female n = 9-11, 4½ months; male n = 5-10, female n = 8-15, 9 months; male n = 16-28, female n = 23-30.

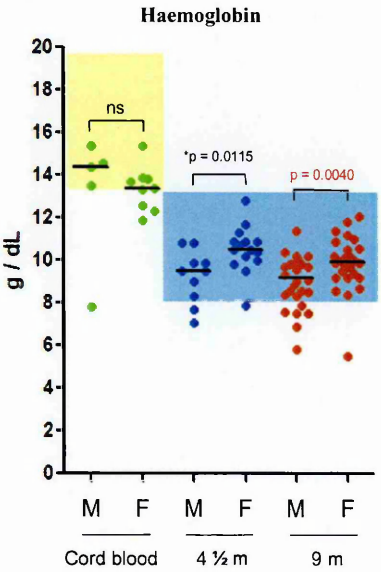
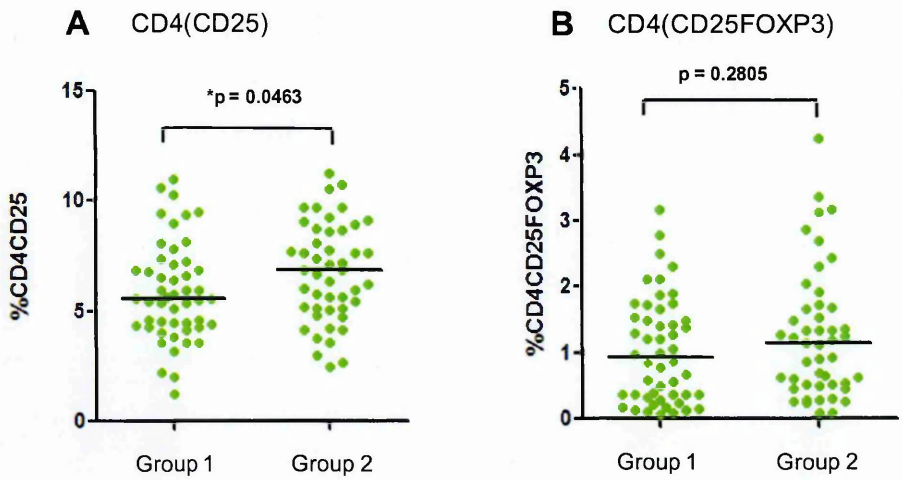


Figure 4.2: Gender differences in haemoglobin levels in each age group. *Haemoglobin levels were determined from 250 µL whole blood using a cell analyser. Cord blood M: n = 5, F: n = 9, 4½ months M: n = 10, F: n = 15, 9 months M: n = 28, F: n = 30. Black bar represents median value and the blue shaded region represents the acceptable range of values from Kilifi, Kenya for children 12 months – 10 years. The yellow shaded region represents the cord blood ranges from Zimbabwe. Mann Whitney U test was applied to compare differences across ages at 5% significance, * represents values that were not significant after adjustment for multiple testing.*

4.2.3 Differences in ex vivo cell phenotypes within the first 9 months of life

Immune cells from whole blood without stimulation (*ex vivo*) were phenotyped at birth (cord blood), 4½- and 9- months of age using flow cytometry with a panel of antibodies that defined activated T cells ($CD4^+CD25^+$) and Tregs ($CD4^+CD25^+FOXP3^+$) as described in Chapter 2: Material and Methods.

Infants were randomised at birth to receive BCG or not. There was no reason to expect differences between the two groups in cord blood prior to BCG vaccination in either group. This was true for the absolute whole blood counts, the $CD4^+/CD8^+$ T cell populations and the $FOXP3^+$ Tregs. However there were significantly more $CD4^+CD25^+$ activated T cells (p = 0.0463) in cord blood from Group 2 than Group 1 although this was not sustained after adjustment for multiple testing (Figure 4.3A - C).



C

	CORD BLOOD		
	Group 1	**Group 2**	***p value***
Lymphocytes (absolute number)¶	138,604	152,024	0.2798
CD4 (%)	38.37	37.57	0.8813
CD8 (%)	19.31	22.83	0.0984
CD4(CD25) (%)	5.58	6.89	*0.0463
CD4(CD25FOXP3) (%)	0.92	1.14	0.2805

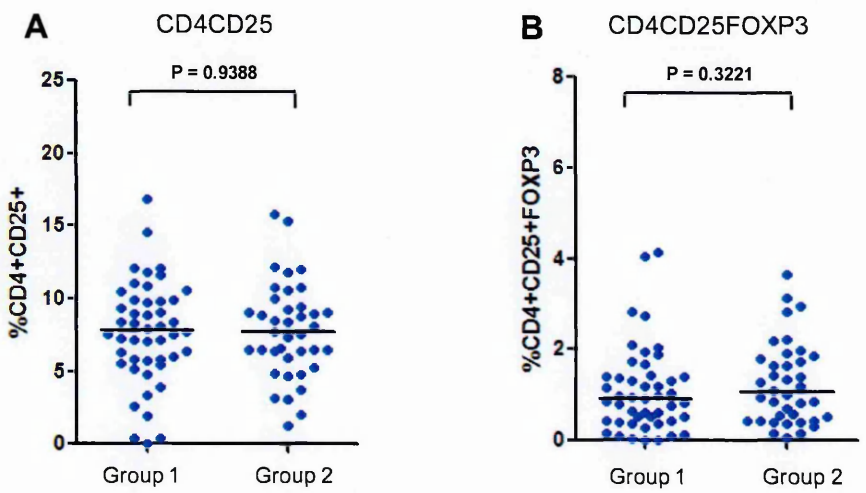
*not significant after Bonferroni correction, ¶ total acquired events was 500,000 for each sample

Figure 4.3: Ex vivo cord blood responses. *100 µl cord blood was phenotyped ex vivo with antibodies to CD4, CD8, CD25, FOXP3 and compared between Group 1 (vaccinated at birth) and Group 2 (vaccinated at 4½ months of age). (A) Activated T cells ($CD4^+CD25^{+}$), (B) Tregs ($CD4^+CD25^+FOXP3^{+}$), and (C) summary table of parameters compared. Mann*

Whitney U test was used to analyse the data at 5% significance. Median value is represented by a black line. n = 42 in each group, represents values that were not significant after adjustment for multiple testing.*

Ex vivo immune cell populations were similar between Group 1 and Group 2 at 4½- and 9- months of age (Figure 4.4A-D) and when comparing T cells 4½ months post BCG vaccination (i.e. 4½ months in Group 1 compared to 9 months in Group 2)(data not shown). However, differences were found when the data was analysed longitudinally.

4½ MONTHS OF AGE



9 MONTHS OF AGE

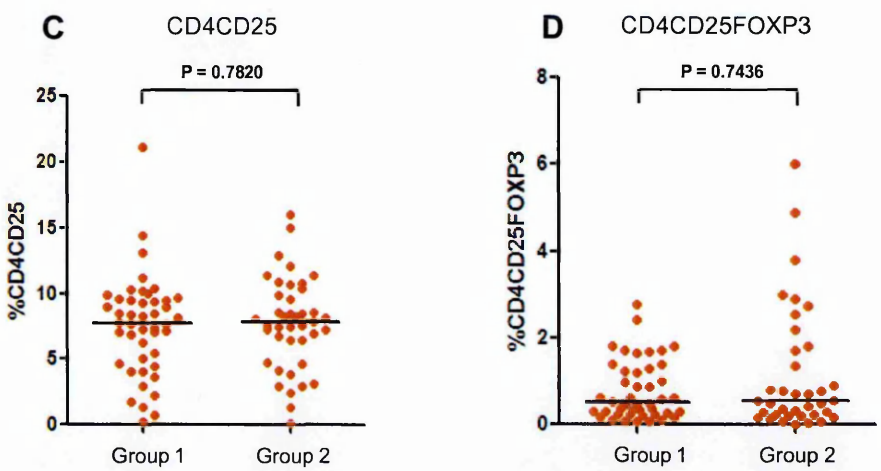
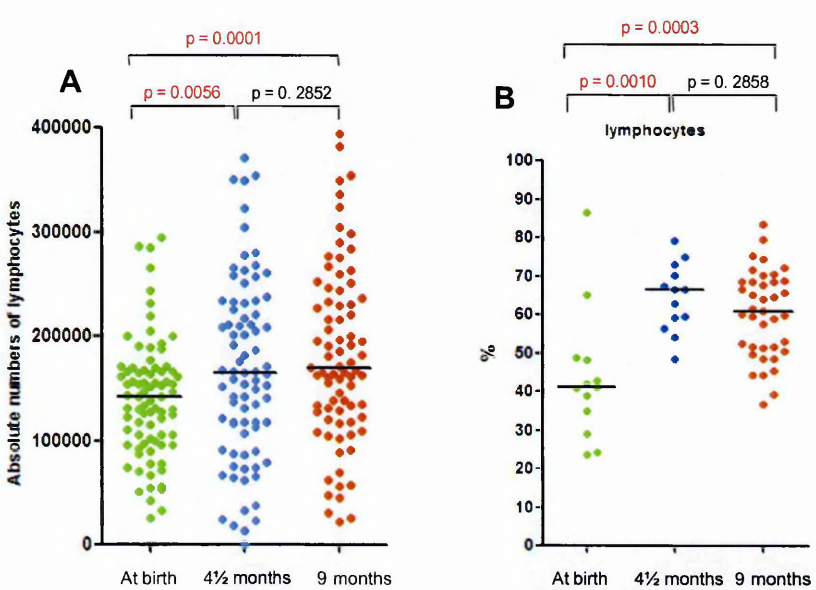


Figure 4.4: Ex vivo T cell populations at 4½- and 9- months of age. *100 µl cord blood was phenotyped with antibodies to CD4, CD8, CD25, and FOXP3. (A) Activated T cells at 4½ months of age, (B) Tregs at 4½ months of age, (C) activated T cells at 9 months of age and (D) Tregs at 9 months of age. A Mann Whitney U test was used to analyse the data at 5% significance. Median value is represented by a black line. 4½ months Group 1 n = 48, Group 2 n = 39; 9 months Group 1 n = 45, Group 2 n = 40, * represents values that were not significant after adjustment for multiple testing.*

From 100 µl blood, 500,000 events were acquired on the FACSCalibur for each sample. The absolute number of cord blood lymphocytes present for every 500,000 events obtained were significantly lower (median 141,839) than those acquired at 4½- (median 165,594, p = 0.0056) and 9- months (median 169,312, p = 0.0001) (Figure 4.5A). This corresponded with the results obtained on the automatic cell analyser (Figure 4.5B). The non-lymphoid populations observed at all ages is likely to consist of macrophages and granulocytes which have greater size and/or granularity (Figure 4.5C) although these cell populations were not analysed in this study.



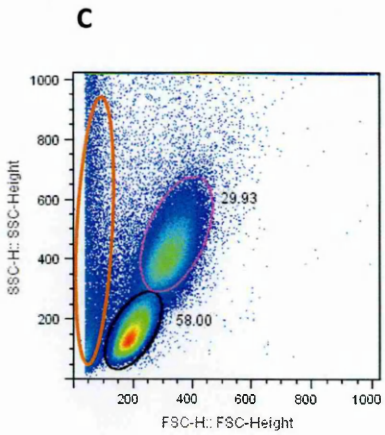


Figure 4.5: Lymphocyte numbers ex vivo in young infants. *100 µl whole blood was phenotyped by flow cytometry immediately after collection. (A) absolute lymphocyte numbers in whole blood compared across ages, n = 81. (B) the percentage of lymphocytes in whole blood compared across ages using a cell analyser, at birth and 4½ months n = 13 and 9 months n = 39. The back bar represents the median value. A Mann Whitney U test was used to analyse the data at 5% significance. (C) representative flow cytometry plot ex vivo at 4½ months showing lymphocyte population (black oval) and the non-lymphoid population (pink oval). In cord blood there are a greater number of dead cells (illustrated in the region of the red oval).*

Paired longitudinal analysis of *ex vivo* immune populations was performed for all individuals grouped together regardless of BCG vaccination status for whom there was data for all three time points (Group1 n = 44, Group 2 n = 37). The percentage of activated T cells increased from birth to 4½ months and remained stable at 9 months, while there was a non-significant trend for reduced Tregs over time (Figure 4.6A and B).

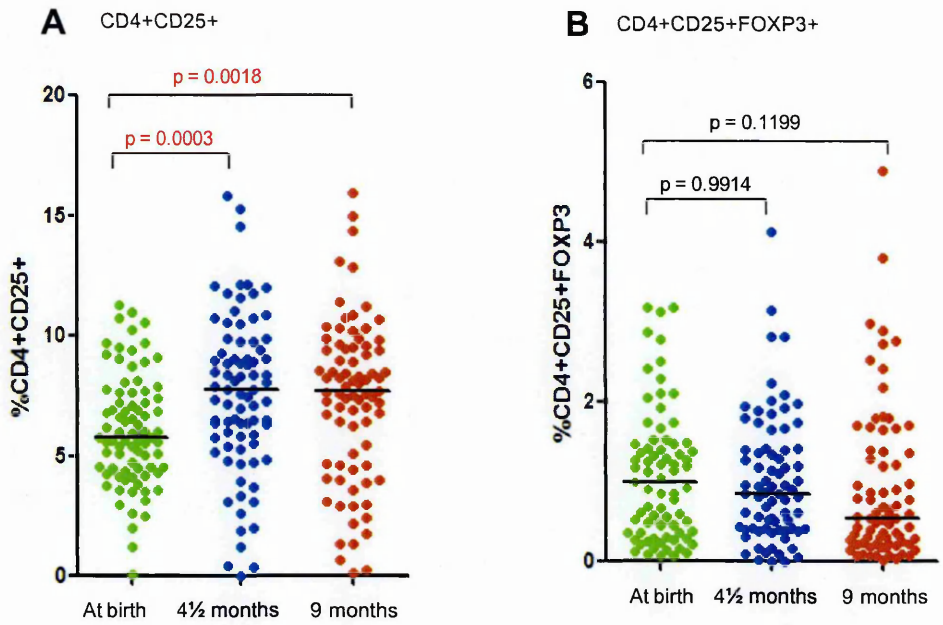


Figure 4.6: Longitudinal analysis on ex vivo phenotypes in all subjects. *100 µl whole blood at each time point was phenotyped with antibodies to CD4, CD8, CD25, and FOXP3. (A) $CD4^+CD25^+$ T cells, n = 81, (B) $CD4^+CD25^+$ $FOXP3^+$ T cells, n = 77. A Wilcoxon non-parametric paired test was used to analyse the data at 5% significance. Median value is represented by a black line.*

When analysing longitudinal changes by vaccine groups, differences in the *ex vivo* phenotype emerged. Compared to cord blood, individuals vaccinated at birth (Group 1) had increased activated T cells at 4½ months (p = 0.0013) that was not observed in BCG naïve infants (Group 2) (Figure 4.7A and B). There were no differences in Group 2 4½ months post BCG vaccine (assessed at 9 months of age) compared to cord blood or at 4½ months of age (Figure 4.7B and D).

Ex vivo $CD4^+$ T cells, $CD8^+$ T cells (Table 4.4) and $FOXP3^+$ Tregs (Figure 4.7C and D) were similar over the 9 months and were not altered by BCG vaccination.

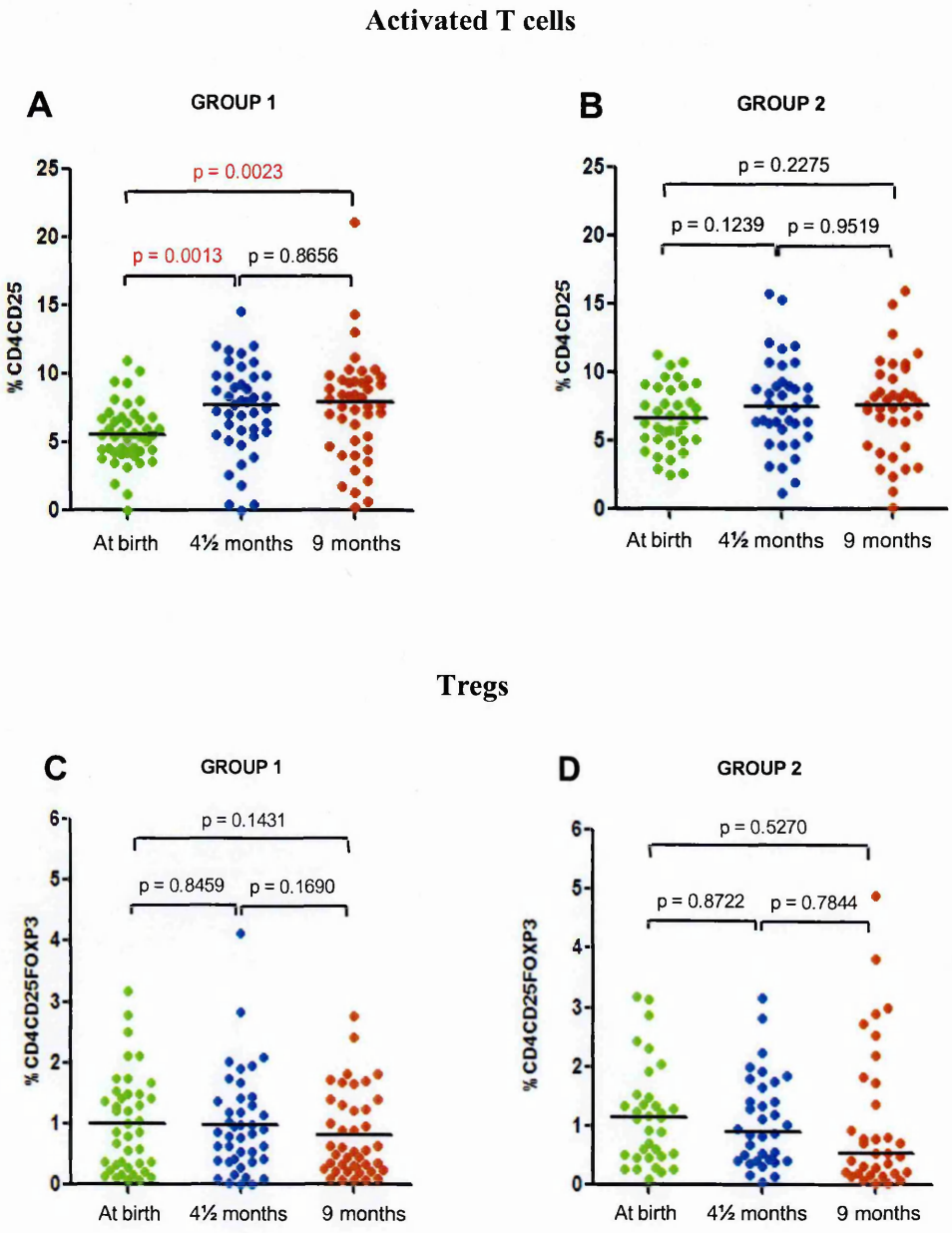


Figure 4.7: Longitudinal analysis on ex vivo phenotypes according to groups. *100µl whole blood at each time point was phenotyped with antibodies to CD4, CD8, CD25, FOXP3. Data was separated into groups according to BCG vaccine schedule; Group 1 = vaccinated at birth, Group 2 = vaccinated at 4½ months. (A) Group 1 $CD4^+CD25^+$ T cells, n = 44 (B) Group 2 $CD4^+CD25^+$ T cells, n = 37, (C) Group 1 $CD4^+CD25^+$ $FOXP3^+$ T cells, n = 42 (D) Group 2 $CD4^+CD25^+FOXP3^+$ T cells, n = 35. A Wilcoxon non-parametric paired test was used to analyse the data at 5% significance. Median value is represented by a black line.*

	At birth	4½ months	9 months	Birth vs 4½ m	Birth vs 9 m	4½ m vs 9 m
lymphocytes	141,839	165,594	169,312	0.0056	0.0001	0.2852
CD4 (%)	38.79	35.87	38.07	0.0779	0.1356	0.3610
CD8 (%)	20.93	17.37	17.25	*0.0102	0.0893	0.8815
CD4(CD25) (%)	5.79	7.74	7.7	0.0003	0.0018	0.8506
CD4(CD25FOXP3) (%)	1.00	0.865	0.54	0.9914	0.1199	0.2267

Table 4.4: T cell populations at different age groups. *100 µl whole blood at each time point was phenotyped with antibodies to CD4, CD8, CD25, FOXP3, cord blood n = 74, 4½ months n = 80, 9 months n = 82. A Wilcoxon non-parametric paired test was used to analyse the data longitudinally at 5% significance, blue text indicates values greater than the earlier time point. *represents values that were not significant after adjustment for multiple testing.*

The reliability of FOXP3 as a marker of human Tregs has been a matter of concern since it can also be transiently upregulated upon activation of T cells (Gavin, Torgerson et al. 2006; Allan, Crome et al. 2007; Popmihajlov and Smith 2008) although this induction consistently peaked at 1 - 3 days of culture and reduced to baseline levels by 5 - 10 days. This would suggest Treg populations observed in this study after 5 days stimulation were likely to be 'true' Tregs (see Chapter 2: Materials and Methods and Appendix VII).

In light of these results a strong correlation between activated and regulatory T cells at 9 months of age was found in both vaccine groups (Table 4.5A and B). A weaker correlation also existed between activated T cells and Tregs in cord blood and at 4½ months although this was not significant after adjusting for multiple testing, and was not apparent when analysing by vaccination group.

It was hypothesised that increased regulatory T cells induced by exposure to environmental mycobacteria within the first 4½ months of life may attenuate the response

to BCG. When vaccinated at birth, the proportion of Tregs prior to vaccination was positively correlated to the levels of activated T cells ($p < 0.0001$, $r = 0.5821$) and Tregs at 9 months ($p < 0.0001$, $r = 0.6150$) but not at 4½ months (Table 4.5A and Figure 4.8). For Group 2 (vaccinated at 4½ months) there was, similarly, no correlation at 4½ months post vaccine ($p = 0.3419$, $r = 0.1708$)(Table 4.5B). The response at 9 months post vaccine in Group 2, i.e. 13½ months of age was not measured in this study.

A

GROUP 1	Tregs at birth	Tregs at 4½ m	Tregs at 9 m
Activated T cells at birth	0.2613	0.3742	0.6883
Activated T cells at 4½ m	0.7968	0.0651	0.7871
Activated T cells at 9 m	< 0.0001	0.2542	0.0001
Tregs at birth		0.0583	< 0.0001
Tregs at 4½ m			0.1732
Tregs at 9 m			

B

GROUP 2	Tregs at birth	Tregs at 4½ m	Tregs at 9 m
Activated T cells at birth	*0.0311	0.6498	0.7494
Activated T cells at 4½ m	0.7054	0.4508	0.8466
Activated T cells at 9 m	0.4543	0.3419	0.0028
Tregs at birth		0.3038	0.6424
Tregs at 4½ m			0.7387
Tregs at 9 m			

Table 4.5: Correlations between $CD4^+CD25^+$ activated T cells and $CD4^+CD25^+FOXP3^+$ Tregs. *100 µl whole blood at each time point was phenotyped with antibodies to CD4, CD25, FOXP3. Comparisons between activated T cells ($CD4^+CD25^+$) and Tregs ($CD4^+CD25^+FOXP3^+$) in (A) Group 1 (vaccinated at birth) and (B) Group 2 (vaccinated at 4½ months of age) were examined using Spearman's correlation coefficient. Values*

*represented are p values. Blue text = significant values at 5% significance. * represents values that were no longer significant after Bonferroni. Activated T cells; Group 1 n = 44, Group 2 n = 37, Tregs: Group 1 n = 42, Group 2 n = 35.*

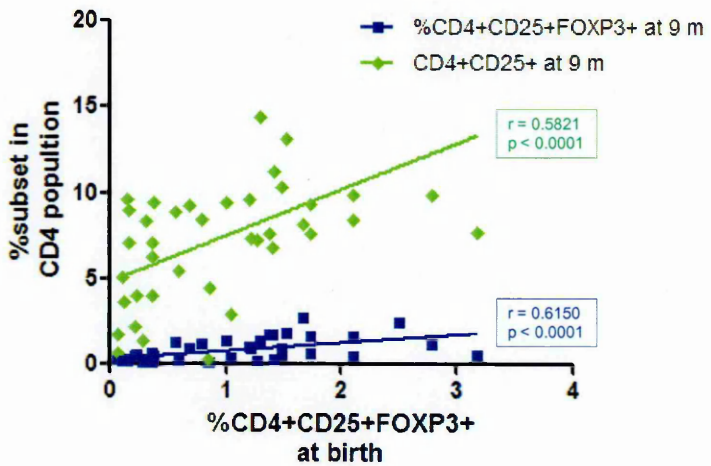


Figure 4.8: Correlation between regulatory T cells at birth and T cell subsets at 9 months in Group 1. *100 µl whole blood at each time point was phenotyped with antibodies to CD4, CD25, FOXP3. Comparisons between Tregs ($CD4^+CD25^+FOXP3^+$) at birth and activated T cells ($CD4^+CD25^+$), n = 44 and Tregs n = 42 at 9 months of age in Group 1, were examined using Spearman's correlation coefficient.*

4.3 DISCUSSION

The reference ranges used for this study were based on values from African populations since Western ranges are not appropriate; for example, the World Health Organisation (WHO) reports anaemia in adults as <13 g/dL for men and <12 g/dL for women (<11 g/dL pregnant women) (WHO and CDC 2007), whereas these levels are within the normal range for an African adult population. The results of the study will contribute to a database of pooled results from Gambian infants to establish appropriate reference ranges in this study population.

As has been documented previously the blood cell indices changed significantly in the first 9 months of life. Cord blood indices were markedly different to those observed at 4½- and 9- months of age. Higher levels of Hb, PCV, MCV and MCH alongside reduced RBCs suggest that the red blood cells in cord blood are larger, denser and contain more haemoglobin per cell compared to infants. This may reflect the larger number of nucleated-RBC precursors (NRBC) present in cord blood. Within a few months of life these are enucleated by the liver and disappear from the peripheral circulation (Rolfo, Maconi et al. 2007). Foetal Hb is both structurally and functionally different to adult Hb and may partially account for markedly higher levels in cord blood. However, because of their size and the ability for these NRBCs to resist lysis they may also be counted as white blood cells in the automatic cell analyser, thus leading to a falsely high white blood cell count. Indeed, recent automated counting methods that can distinguish these cells from normal white blood cells have demonstrated that approximately 3% (0 – 11.6%) of WBC in cord blood are in fact NRBC (Rolfo, Maconi et al. 2007). This may well explain the higher numbers of cells classified as WBCs in cord blood in this study and the higher reference ranges for WBCs in cord blood (Figure 4.1 and Table 4.2). The lymphocyte population showed the opposite trend to WBC and was lowest at birth increasing with age. Thus lymphocytes cannot account for the higher WBC count at birth. Increased WBC often accompanies infections but none of these children exhibited signs of infection when

assessed at birth. Subclinical infections such as CMV or Epstein-Barr virus (EBV) can be acquired very young (including congenitally) in this population and are often asymptomatic (Miles, van der Sande et al. 2007) and may contribute to this finding, however all those with high WBC values at birth were not infected with CMV (data not shown).

Many studies have shown gender differences in blood cell indices in adults, but there are conflicting views as to whether the differences are physiological or are due to hormonal changes in women (Jackson, Carter et al. 2001; Rushton, Dover et al. 2001).The higher levels of Hb, MCV and MCH observed in 9 month old females compared to males in this study, is in contrast to patterns observed in adult studies where males have the higher blood cell indices suggesting some gender differences may not be related to sex hormones. It was also found that 80% of anaemic infants at 4½- and 9- months of age were boys. In support of our findings, Meinzen-Derr *et al* found that in a Mexican cohort, 65% of anaemic children at 9 months of age (Hb < 10g/ dL) were boys (Meinzen-Derr, Guerrero et al. 2006). Interestingly this study also showed that infants who are exclusively breast-fed for >6 months of age in developing countries may be at an increased risk of infant anaemia (Meinzen-Derr, Guerrero et al. 2006). In addition, extrapolated data from a study in Gambia found that the higher range of Hb for females up to 15 years of age than males but this reversed from 15 years to adult ranges where males have the higher ranges (Adetifa, Hill et al. 2008). There are reports that various vaccine regimes in early life can influence morbidity and mortality in a gender related fashion (Aaby, Jensen et al. 2003; Garly, Jensen et al. 2004; Veirum, Sodemann et al. 2005; Aaby, Ibrahim et al. 2006; Aaby, Biai et al. 2007), further supporting gender effects at the pre-pubertal stage in life. Further gender related immune cell activity will be discussed in Chapter 7.

Unlike mice, human T cell production occurs during gestation and therefore at birth

a diverse repertoire of T cells are present, although 90% of these are thought to have a naïve phenotype ($CD45RA^+$) (Adkins, Leclerc et al. 2004). This study analysed, by flow cytometry *ex vivo* lymphocytes expressing the markers CD4, CD8, CD25 and FOXP3.

Flow cytometry confirmed lower lymphocyte levels in cord blood compared to the other ages as seen with the cell analyser results. This agrees with published studies suggesting that they exhibit a higher degree of apoptosis compared to adults (Szabolcs, Park et al. 2003; Thornton, Upham et al. 2004; Kessel, Yehudai et al. 2006). $CD4^+$ and $CD8^+$ T cell frequencies within the lymphocyte population were comparable across all three age groups although they were reduced compared to the UK but similar to those observed from studies in India, Botswana and China (Das, Bhanushali et al. 2008). This further highlights the importance of geographical location in determining the 'normal' range for immune cell subsets.

One of the main aims of the study was to analyse the effect of delaying BCG vaccination on immune development. It was hypothesised that exposure to NTM in the absence of BCG vaccination might lead to the induction of Tregs. Certainly there was no difference in *ex vivo* Tregs at 4½ months of age between BCG vaccinated (Group 1) and BCG naïve (Group 2) infants suggesting Treg induction in the peripheral circulation was not affected by the presence or absence of BCG vaccination. Antigen specific responses will be discussed in later chapters.

By contrast $CD4^+CD25^+$ T cell frequencies did increase significantly from birth in the vaccinated group, but not in the BCG naïve group. However there was no difference between groups when comparing responses at 4½ months of age which may be due to the lower activated T cells in the group vaccinated at birth (Group 1).

The strong positive correlation between Tregs prior to vaccination at birth and both activated T cell and Treg frequencies at 9 months of age is difficult to explain but suggest a relationship does exist between immune status at the time of receiving BCG and the *ex vivo* immune 'set point' 9 months later. However this effect was not strong enough to lead

to statistical differences between Tregs or activated T cells in this Group 1 (vaccinated at birth) and those vaccinated 4½ months later.

Ex vivo responses may be confounded by several things including timing of previous vaccines, illness or external factors. These confounders were minimal in our study as the two groups were under the same environmental pressures and blood was not collected from children who exhibited signs of intercurrent infection. As mentioned previously, chronic asymptomatic infections such as CMV, EBV or colonisation by helminth parasites could all lead to changes in circulating T cells. In this community, previous studies have estimated 90% of children are infected with CMV by 12 months of age (Miles, van der Sande et al. 2007). CMV infection status of the children in this study will be discussed in chapter 7. Helminths, however seem to be less common in Gambia than expected. Nyan *et al* found 17% and 8.2% helminth infection in adults from urban and rural locations, respectively (Nyan, Walraven et al. 2001). More recent studies have shown very low levels, between 0 – 3% in adult populations from both low and high malaria exposed regions suggesting helminth infection is unlikely to be a confounding factor (Finney 2009).

Overall, cord blood exhibits different ranges for blood cell indices compared to infants at 4½- and 9- months which may largely be due to NRBCs present in cord blood but the differences observed in T cell populations were less obvious. Circulating activated T cells increased with age and possibly after BCG vaccination at birth. It was also concluded that the level of Tregs prior to BCG vaccination may relate to the frequencies of circulating activated T cells and Tregs present 9 months later.

CHAPTER 5

Exposure to environmental mycobacteria influences immunogenicity of BCG

5.1 INTRODUCTION

In countries that are high risk for TB, BCG is recommended to be given at birth, on first contact with healthcare workers, or at least within the first year of life, although there is little immunological evidence that confirms when best protection is achieved. BCG at birth induces quantitatively and qualitatively similar Th1 responses to vaccination as an adult, although many other vaccines (HBV, OPV, measles) given early in life induce Th2 responses (reviewed in (Marchant and Goldman 2005)). The BCG induced Th1 response is thought to be due to mycobacteria being potent activators of dendritic cells (Henderson, Watkins et al. 1997; Ausiello, Fedele et al. 2002). However, there is evidence that presentation of antigens is less efficient in neonates (reviewed in (Fadel and Sarzotti 2000) and (Siegrist 2001)) and, although TLR expression patterns on neonatal innate cells are similar to adults, they have reduced function (reviewed in (Levy 2007)).

5.1.1 Role of cytokines in BCG immunogenicity

Immune correlates of protection against TB have mainly focused on T cell production of IFNγ (Ellner, Hirsch et al. 2000). The importance of this cytokine can be demonstrated in those individuals with polymorphisms of IFNγ or those with IFNGR deficiency. Patients with both dominant and recessive deficiencies in IFNγR1 and STAT1 have increased mycobacterial susceptibility and in the case of recessive STAT1 mutations, disseminated BCG disease can develop after BCG vaccination (reviewed in (Rosenzweig and Holland 2005)). Induction of IFNγ is associated with IL-12 production and therefore it is not surprising that deficiencies in IL-12 can also exhibit themselves in susceptibilities to mycobacterial infection and impairment of memory T cell functions (Cleary, Tu et al. 2003).

BCG induces a strong Th1 response, in particular the production of IFNγ, but can also induce other cytokines that may affect the protective response. A Th2 response is often evident alongside the Th1 cytokine production in response to BCG, the dynamics of which

may be important in the maintenance and subsequent protection levels that are achieved. Sander *et al* showed that 1 – 2 days after *in vitro* stimulation with live BCG the response is dominated by low IFNγ and TNFα production. By 4 – 5 days there was a marked production of Th1 lymphokines with 6% $IFN\gamma^+$, 4% $TNF\alpha^+$ and 2% $IL\text{-}2^+$ producing T cells. Late in the reaction, at 10 – 12 days, a Th2 response with increased IL-4, IL-5 and IL-10 was detected while the synthesis of Th1 cytokines declined (Sander, Skansen-Saphir et al. 1995). The protective effect of BCG-induced IFNγ has also come into question recently. Using 3 different *in vitro* bacterial inhibition assays, *M.tb* lysate or live BCG growth inhibition did not correlate with IFNγ production from BCG stimulated 5 day cultures (Hoft, Worku et al. 2002). In addition a recent human study in South Africa showed that a significant number of BCG induced CD4 T cells failed to express IFNγ, but expressed TNFα and IL-2 (Soares, Scriba et al. 2008). These studies suggest that BCG-induced IFNγ may not be sufficient to study BCG protective responses. As reported in studies of TB both in mice and humans (reviewed in Chapter 1) the lack of IFNγ does not always correspond to lack of protection suggesting a signature comprising of a number of cytokines may be more informative as a correlate of protection. IL-17 is a pro-inflammatory cytokine that is predominantly produced by Th17 differentiated $CD4^+$ T cells in the presence of IL-23, (Lockhart, Green et al. 2006; Khader, Bell et al. 2007; Khader and Cooper 2008). TGFβ together with IL-6 or IL-21, independently of IL-23 can also initiate this production suggesting a link with the innate response (Awasthi and Kuchroo 2009). Interestingly, without IL-6 production, TGFβ initiates development of adaptive Th3 Tregs and therefore these cell types may be closely regulated acting optimally to control infection (Weaver, Harrington et al. 2006). Tuberculosis patients have shown reduced levels of $CD4^+$ production of IL-17 suggesting Th17 may be protective against TB (Scriba, Kalsdorf et al. 2008; Sutherland, Adetifa et al. 2009). It has been proposed that BCG vaccination induces IL-17-producing $CD4^+$ T cells that populate the lungs and, after challenge, trigger the production of chemokines that recruit $CD4^+$ T cells producing IFNγ

which ultimately restrict bacterial growth (Khader, Bell et al. 2007). However IL-17 is increased in IFNγ deficient mice following infection with BCG and these mice were unable to control the infection suggesting that IL-17 is not protective or that interaction with IFNγ is required for IL-17 function (Cruz, Khader et al. 2006). IL-23 plays a role in generating IL-17 producing T cells and in the absence of IL-23, BCG vaccination of mice did not induce an accelerated memory response suggesting IL-23 may be required as part of the priming of a secondary response rather than during the initial effector priming (Khader, Bell et al. 2007).

It has long been recognised that suppressor responses can also be induced by BCG (Bennett, Rao et al. 1978; Schrier, Allen et al. 1980) but more recently nTregs (Jaron, Maranghi et al. 2008) and IL-10 production (possibly by aTregs) have been shown to play a role, both in animal models (Jacobs, Fick et al. 2002; Barlan, Bahceciler et al. 2006; Morel, Badell et al. 2008; Li and Shen 2009) and in humans (Hanekom 2005; Sendide, Deghmane et al. 2005; Madura Larsen, Benn et al. 2007; Mendez-Samperio, Trejo et al. 2008). As mentioned above, the dynamics of cytokine induction following BCG vaccination has been shown to vary according to cytokine in *in vitro* studies, and this may correspond to the development of anti-mycobacterial immunity. Thus, Nabeshima *et al* showed that the IFNγ response to PPD in adults vaccinated with BCG, peaked at 8 weeks and declined by 12 weeks, whereas IL-10 levels peaked at 2 weeks and declined to the lowest point 8 weeks after stimulation (Nabeshima, Murata et al. 2005).

5.1.2 Innate response to BCG

More recently it has been recognised that, in mice BCG can infect cells of the innate immune system (neutrophils, DCs and NK cells) often through TLRs leading to a polarised adaptive response indicating that mechanisms which upset these early interactions could alter T cell immunity to BCG vaccination (Esin, Batoni et al. 2004; Feinberg, Fieschi et al. 2004; Madura Larsen, Benn et al. 2007; Naoe, Ogawa et al. 2007;

Marcenaro, Ferranti et al. 2008; Morel, Badell et al. 2008). It is likely that the majority of IFNγ responses early in infection are produced by NK cells (Esin, Batoni et al. 2004; Feinberg, Fieschi et al. 2004; Marcenaro, Ferranti et al. 2008). Receptor NKp44 was induced after 3 and 4 days of *in vitro* stimulation with live BCG suggesting BCG directly interacts with NK cells (Esin, Batoni et al. 2008). In addition complement has also been shown to play a role in BCG immunogenicity. Complement C5a (anaphylatoxin), secreted by mycobacteria-infected macrophages, regulates IL-12p70 production. DCs from mice that lacked C5a secreted less IL-12p70 and more IL-10 in response to BCG, which led to reduced IFNγ production from $CD4^+$ and $CD8^+$ T cells (Moulton, Mashruwala et al. 2007).

5.1.3 Role of Tregs in BCG immunogenicity

Recent interest in regulatory T cells has led to the idea that Tregs (both nTregs and aTregs) may play a role in the anti-mycobacterial immunity. One investigation examined whether nTregs suppress the development of protective immunity to BCG in mice. Inactivating $CD4^+CD25^+$ Tregs prior to BCG vaccination increased the numbers of IFNγ producing $CD4^+$ and $CD8^+$ T cells from the draining lymph nodes at 14 days post vaccination but surprisingly, protection was not affected (Quinn, Rich et al. 2008). The authors proposed this may have been due to an induction of aTregs in the periphery and/or IL-10 production but also support the increasing evidence that IFNγ is not the sole cytokine responsible for protection.

5.1.4 BCG immunogenicity in humans

Human studies of infant BCG vaccination have shown varying degrees of IFNγ responses which seem to be dependent on the timing of vaccination, as reported in cattle studies (Buddle, Wedlock et al. 2003). Marchant *et al* found that delaying the BCG vaccination to 2 months induced a trend for lower PPD stimulated IFNγ production 2 months later compared to vaccination at birth although this was not significantly different.

Overnight IFNγ responses to PPD-tuberculin (PPD-T) persisted to 12 months of age in all vaccine groups (vaccination at birth, 2- and 4- months of age) although lower than initial T cells responses, suggesting memory T cells were activated (Marchant, Goetghebuer et al. 1999). In contrast to this work, delaying the BCG vaccine to 10 weeks of age and assessing 10 weeks later, showed a trend for a greater induction of both IFNγ and IL-10 production in response to heat killed BCG after 5 days stimulation compared to 10 weeks post vaccination at birth although this, also was not statistically significantly. In addition the sample size was small (n = 6 – 11) and therefore there is a high probability of this trend occurring by chance (Hussey, Watkins et al. 2002).

5.1.5 NTM effect on BCG immunogenicity

It has been suggested that the variation in BCG efficacy across different geographic locations (Rodrigues, Diwan et al. 1993; Colditz, Brewer et al. 1994; Colditz, Berkey et al. 1995) is due to attenuated responses following exposure to NTM (the contribution of which varies across the globe) (Palmer and Long 1966; ten Dam 1984; Fine, Sterne et al. 1994). Epidemiological studies have illustrated greater efficacy of BCG in neonates vaccinated before prior NTM exposure (Colditz, Brewer et al. 1994; Colditz, Berkey et al. 1995) (Tidjani, Amedome et al. 1986; Lanckriet, Levy-Bruhl et al. 1995) and in vaccine trials where TST positive individuals have been excluded (Hart and Sutherland 1977). Areas with high exposure to NTM, like Southern India, where isolates of *M. avium-intracellulare-scrofulaceum* (MAIS) are commonly found in water and dust (Kamala, Paramasivan et al. 1994), BCG is particularly ineffective (Fine 1995). Animal studies have also provided support for this hypothesis. The original work was performed in guinea pigs sensitised with *M. fortuitum, M. avium* and *M. kansasii* prior to BCG vaccination. Palmer and Long suggested that environmental (non-tuberculous) mycobacteria (NTM) exposure primed the immune response providing a level of protection that then masks the protective response from BCG vaccination (Palmer and Long 1966). A second theory suggests that

exposure to NTM blocks the BCG growth and protection. Mice and guinea pigs pre-sensitized with *M. avium* or with cocktails of *M. avium, M. vaccae,* and *M. scrofulaceum* develop anti-mycobacterial responses that control the multiplication of BCG, thereby reducing its protective efficacy against TB (Kamala, Paramasivan et al. 1996; Brandt, Feino Cunha et al. 2002). The blocking of BCG immunogenicity by *M. avium* has been observed in mice and cattle by several research groups (Brandt, Feino Cunha et al. 2002; Young, Slobbe et al. 2007).

Cattle studies have also been useful in the understanding of the immunogenicity of BCG; they are the natural host for *M. bovis* and the clinical disease processes are very similar to humans. It also provides a model of natural sensitisation of NTM which can be exploited to consider its effect on BCG vaccination. Calves have minimal responsiveness to NTM antigens at birth but by 6 – 9 weeks of age most are responding to PPDs prepared from *M. avium* and *M. bovis* and some to ESAT-6/ CFP-10 antigens in a overnight whole blood IFNγ assay (Buddle, Wedlock et al. 2003). Delaying BCG vaccination to 6 weeks of age compared to at birth induced similar IFNγ responses, but less proliferation in response to PPD, although the level of protection conferred was similar (Buddle, Wedlock et al. 2003). Interestingly revaccination at 6 weeks after prior vaccination at birth reduced protection in calves possibly by inducing pro-inflammatory immunopathology (Buddle, Wedlock et al. 2003). However when comparing this study to an earlier study from the same group, vaccination at birth or 6 weeks of age was better at protecting calves from disease compared to vaccination at 5 - 8 months of age (Buddle, Wards et al. 2002). This suggests that the dynamics of NTM exposure at different ages is different and therefore the age of vaccination is important in the relationship between the response to NTM antigens and protection by BCG. Theories of how NTM may affect BCG immunogenicity are discussed later but briefly it is thought that exposure to mycobacterial antigens similar to that of BCG masks or inhibits the response to BCG.

The mechanism by which pre-sensitisation with NTM reduces the protective capacity of BCG may be due to priming of the host immune system against mycobacterial antigens shared with BCG, and that recall of this immune response upon vaccination results in accelerated clearance of BCG and hence decreased protective immunity against TB. Another possibility is that early exposure to cross-reactive mycobacteria may imprint an inappropriate Th2 or regulatory T cell response which negatively influences the response to BCG (Rook, Bahr et al. 1981; Stanford, Shield et al. 1981; Young, Slobbe et al. 2007). However, when mice were pre-sensitised with a more phlogenetically distant mycobacteria such as *M. vaccae*, the protective efficacy was enhanced, suggesting that strain variation and genetic relatedness of the mycobacteria can affect immune responses to BCG (Demangel, Garnier et al. 2005). Thus prior exposure to certain mycobacteria can prime the immune response to mycobacterial antigens and boost the BCG affect but only when it can override the immunological control imparted by sensitisation with these strains (Demangel, Garnier et al. 2005). The blocking or masking of BCG protection by NTM has been shown in several other animal studies (Weiszfeiler and Karasseva 1981; Edwards, Goodrich et al. 1982; Brown, Brown et al. 1985; Orme and Collins 1986; Kamala, Paramasivan et al. 1996; Howard, Kwong et al. 2002).

There are relatively few human studies; most based on the comparisons of populations of high NTM exposure e.g. Malawi, to low exposed areas e.g. UK (Farhat, Greenaway et al. 2006; Weir, Black et al. 2006). Black *et al* showed in Malawi that IFNγ production to PPD following BCG was greater in those individuals with low initial responsiveness to MAIS antigens (Black, Dockrell et al. 2001). These pre-vaccination cytokine responses, from the supernatant of 6 day whole blood assays, were dependent on the type of NTM, where more individuals responded to the MAIS antigens than to other NTM antigens, such as *M. fortuitum* (Black, Dockrell et al. 2001) supporting previous skin test studies in the same population (Fine, Floyd et al. 2001). This finding also suggests that, in Malawi the predominant NTM in the environment may be responsible for the low

protective levels of BCG found in this location. In a separate study by the same group 61% of BCG naïve adults in Malawi were responders to PPD (> 62 pg/mL) compared to 22% in the UK suggesting there is less NTM exposure in the UK than in Malawi. The magnitude of response from before, to 1 year post-vaccination was higher in the UK than Malawi which corresponds to the level of protection observed in these countries (Black, Weir et al. 2002).

In a comprehensive review of the literature, Stanford and Rook *et al* proposed a two-pathway theory postulating that BCG vaccination can trigger either a protective or an antagonistic immune reaction based on the mycobacterial source of pre-vaccination sensitivity (Rook, Bahr et al. 1981; Stanford, Shield et al. 1981).

5.1.6 Non-specific effects of BCG

There are increasing, but still controversial reports that BCG vaccination may influence the maturation of the immune system non-specifically and have impacts on health later in life. It is clear that when administered at birth, BCG protects against leprosy, meningitis and extrapulmonary forms of TB (Antas and Castello-Branco 2008) but there is also evidence that BCG protects against various inflammatory or autoimmune diseases such as allergy, asthma, Crohn's disease, insulin-dependent diabetes mellitus and specific cancers (epidemiological review in (Rousseau, Parent et al. 2008). The non-specific immunostimulatory effects of BCG have been widely documented as a treatment for bladder cancer (Bohle, A *et al*, 2003, Suttmann, H *et al*, 2004, Chen, X *et al*, 2007). BCG has been shown to induce neutrophils to release TNF-related apoptosis-inducing ligand (TRAIL) in an IFNγ dependent manner which then migrates to the bladder and kills cancerous cells (Simons, O'Donnell et al. 2008). In a mouse asthma model BCG induced $CD4^{+}CD25^{+}$ Tregs and FOXP3 expression, accompanied by IL-10 and TGFβ production, and reduced asthma (Li and Shen 2009). It is therefore not surprising that BCG at birth can affect responses to other vaccines in early life (Ota, Vekemans et al. 2002). It was

unexpected that BCG could promote both Th1 and Th2 responses to unrelated antigens. This vaccine adjuvant effect has been attributed to the effect of defective DC maturation in infants. Under suboptimal conditions of co-stimulation of DCs IL-12 has been shown to induce Th1 and Th2 cytokines by neonatal $CD4^+$ T cells (Ota, Vekemans et al. 2002). However further studies are still needed to understand these mechanisms.

In some areas of the world BCG protection is low but can protect against more severe forms of TB as mentioned earlier, such as meningitis and disseminated TB and may persist for 10 years after infant vaccination (meta analysis (Colditz, Berkey et al. 1995)). More recently there is mounting evidence that BCG effects all-cause child morbidity and mortality (Kristensen, I *et al*, 2000, Elguero, E *et al* 2005, Stensballe, LG *et al*, 2005, Roth, A *et al*, 2005, Moulton, LH *et al*, 2005, Rodrigues, A *et al*, 2006). This may influence decisions regarding infant vaccine schedules in the future. With the beneficial effects from severe forms of TB during infancy and the potential for non-specific protection, new strategies for TB vaccines are often based on improving the existing BCG vaccine (e.g. recombinants BCG) or using BCG as the mycobacterial prime followed by a heterologous boost rather than replacing the BCG vaccine completely (reviewed in (Sander 2007)). Non-specific effects of BCG in our study are addressed in Chapter 7.

5.1.7 Heritability of mycobacterial responses

Responses in humans are very heterogeneous necessitating large sample sizes to account for the variation in responses. An interesting study from The Gambia found that in 2 month old, BCG vaccinated infants, IFNγ, IL-13 and IL-5 responses to various mycobacterial antigens (PPD, Ag85, STCF and *KM.TB*) varied within a population by 10-fold (Finan, Ota et al. 2008). Response patterns of responders and non-responders, based on detection levels of cytokine ELISA assays, differed for each cytokine; there were more IFNγ responders than for the Th2 cytokines as expected. It was suggested that this variation within populations might be genetically linked (Newport, Awomoyi et al. 2003;

Finan, Ota et al. 2008). Newport *et al*, 2004 showed that responses to BCG were predominantly controlled by genes within the human leukocyte antigen (HLA) class II locus. Heritability was observed for IFNγ and IL-13 responses to PPD but not to Ag85 or PHA which may be contributed to the promiscuity of the Ag85 peptide that can be presented by multiple MHC class II molecules (Newport, Goetghebuer et al. 2004). Although IFNγ polymorphisms have been associated with clinical TB disease, in the Gambian population IFNγ receptor 1 polymorphism was not associated with pulmonary TB (Awomoyi, Nejentsev et al. 2004). This area of research needs further investigation to understand the contribution genetics plays to BCG responses and protection from TB infection and disease.

This chapter investigates the effect of BCG vaccination early (Group 1, at birth) or delayed (Group 2, at 4½ months of age) on the development of mycobacterial immunity in early life. Blood samples were collected at birth (cord blood), 4½- and 9- months of age. At 4½ months of age comparisons between BCG vaccinated children with BCG naïve children were assessed.

5.2 RESULTS

5.2.1 Reactivity to mycobacterial antigens in cord blood

At birth neither groups had been vaccinated with BCG therefore results were analysed collectively to examine cord blood responses. Cord blood memory responses were assessed comparing unstimulated background reactivity to antigen stimulated values.

Cord blood is thought to contain predominantly naïve T cells and it was therefore surprising that cord blood T cells responded to mycobacterial antigens. Reactivity to PPD was stronger than to BCG, although similar trends were observed. There was no increase in activated T cells when stimulated with each of the mycobacterial antigens tested, but $CD4^+$ and $CD8^+$ T cells proliferated and $FOXP3^+$Tregs increased in response to PPD (Figure 5.1A-C). Proliferation, but not increased Tregs, was also observed with BCG and ESAT-6/ CFP-10 fusion protein stimulation. This is most likely to be predominantly due to $CD4^+$ T cells since after adjustment for multiple testing $CD8^+$ proliferation was no longer significantly increased.

Of the cytokines tested from the supernatants, IFNγ and IL-10 were upregulated compared to unstimulated control for all antigens (*IFNγ*: PPD and BCG $p < 0.0001$, EC $p = 0.0292$, *IL-10:* PPD $p < 0.0001$, BCG $p = 0.0021$, EC $p = 0.0266$) although levels produced were very low. Figure 5.1D illustrates the production of cytokines in PPD stimulated cultures after subtracting the unstimulated controls. Although the levels are low it is evident that there are individuals producing IFNγ and IL-10. In the case of IL-10 in particular, there are a number of individuals who also downregulated IL-10 production (Figure 5.1D). Increased IL-10 production was also observed in response to PPD by flow cytometry ($p = 0.0154$) but not from $CD4^+$ ($p = 0.1014$) or $CD8^+$ ($p = 0.3476$) T cells. IL-10 is produced from many cell types including B cells, monocytes, macrophages and possibly other innate immune cells, but these cells were not analysed in this study. IL-13 production was very low, with the maximum above background being 10 pg/ml, but it was significantly greater than zero ($p = 0.0003$). Adjustment for multiple testing did not alter

any of these findings.

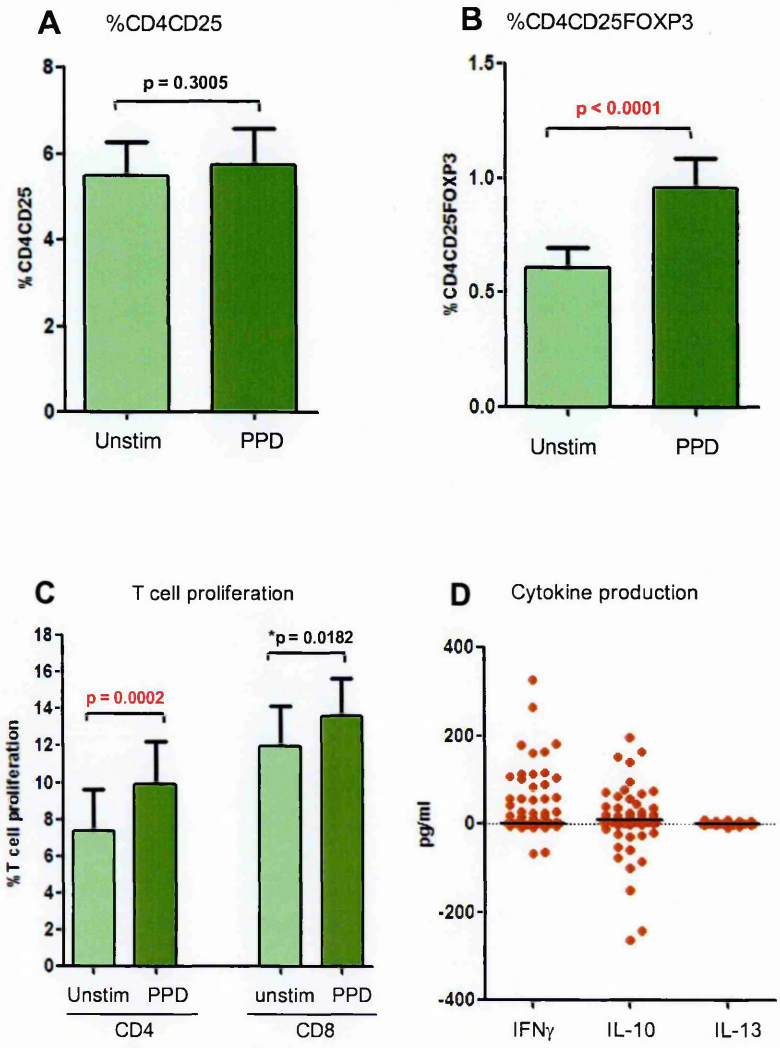


Figure 5.1: In vitro cord blood responses to PPD. *500 µL whole blood diluted 1: 5 was cultured with PPD for 5 days. Comparisons between unstimulated (pale green bars) and PPD stimulated (dark green bars) of $CD4^+CD25^+$ (A), $CD4^+CD25^+FOXP3^+$ (B) and Ki-67^+ (proliferating) T cells (C) in cord blood. Bars represent means and standard error of the means (SEM) error bars. Unstimulated values subtracted from PPD stimulated values were plotted for three cytokines, IFNγ, IL-10 and IL-13 from the culture supernatants (D); median values are presented as black lines. A Wilcoxon non-parametric paired test was*

*applied at 5% significance. Sample number was 76 +/- 4 for flow cytometry and n = 88 for cytokine data. * not significant after Bonferroni correction for multiple testing.*

It was surprising to find that there were responses to ESAT-6/CFP-10 fusion protein (EC) in cord blood above background although the levels were very low. EC induced $CD4^+$ and $CD8^+$ T cell proliferation (Figure 5.2A). In addition, unadjusted values would suggest there was also EC specific IFNγ and IL-10 production (Figure 5.2B and C); the IL-10 production was likely to be from $CD4^+$ T cells (Figure 5.2C) suggesting previous priming from these mycobacterial antigens *in utero*. The EC status of the mothers was not studied but it would be interesting to compare these with the children that responded to the EC antigen.

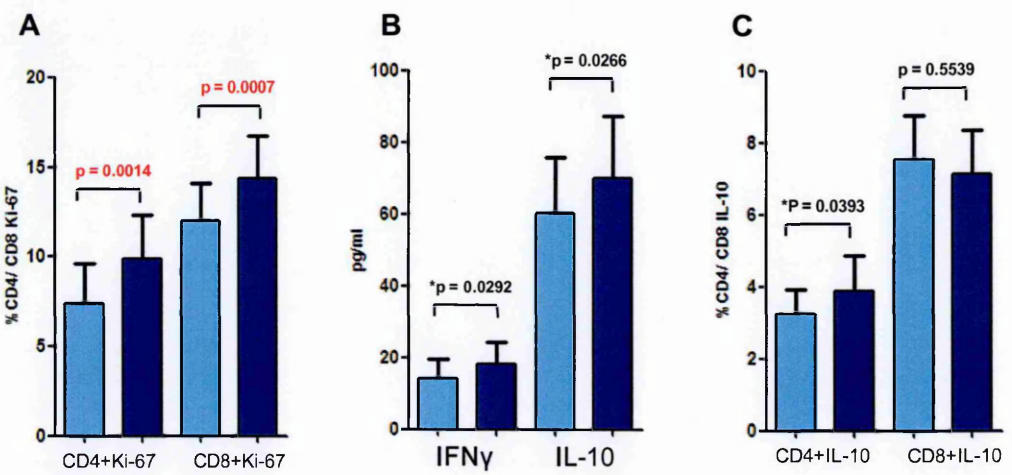


Figure 5.2: Cord blood responses to ESAT-6/CFP-10 fusion protein. *500 µL whole cord blood diluted 1: 5 was cultured with EC for 5 days. Comparisons between unstimulated (pale blue bars) and EC stimulated (dark blue bars) cultures were compared for $CD4^+$Ki-67^+ or $CD8^+$KI-67^+ proliferating T cells (A), IFNγ and IL-10 cytokine production from supernatants (B) and IL-10 production from $CD4^+$ or $CD8^+$ T cells (C). Bars represent means and SEM error bars. Wilcoxon non-parametric paired test was applied at 5%*

*significance. Sample number was 76 +/- 4 for flow cytometry and n = 88 for cytokine data. * not significant after Bonferroni correction for multiple testing.*

5.2.2 Reactivity to mycobacterial antigens at 4½ months of age

At 4½ months there was the opportunity to compare immunity in a BCG experienced group (Group 1) with BCG naïve children (Group 2). Vaccination with BCG at birth elicited activated immune responses 4½ months later in response to PPD. The pattern of responses to BCG was similar but lower, so much of the subsequent analysis discusses PPD reactivity which is consistent with most published work assessing BCG immunogenicity.

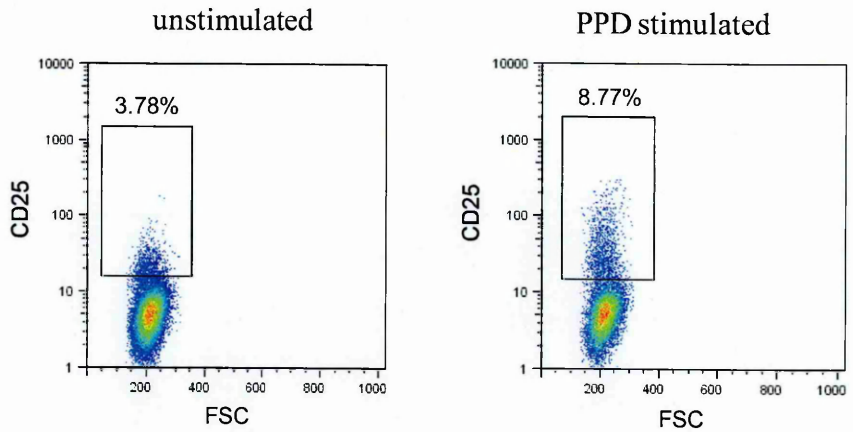
After adjustment for multiple testing it was evident that the BCG vaccinated group exhibited a strong PPD-specific reactivity including increased $CD4^+CD25^+$ activated T cells and $CD4^+FOXP3^+$Tregs in addition to T cell proliferation. In addition there was a down regulation of $CD3^+$ and $CD4^+$ expression by T cells following PPD stimulation (Table 5.1 and Figure 5.3).

	UNS	PPD	*p value*	BCG	*p value*
CD3	61.52	59.24	0.0012	63.01	*0.0291
CD4	44.65	36.85	0.0012	41.24	0.0058
CD8	13.27	15.19	0.0020	14.14	*0.0203
CD4(CD25)	4.19	19.85	< 0.0001	14.53	< 0.0001
CD4(CD25FOXP3)	0.31	7.47	< 0.0001	4.195	< 0.0001
Ki-67	1.43	7.26	< 0.0001	4.25	< 0.0001
CD4(Ki-67)	1.83	11.53	< 0.0001	6.115	< 0.0001
CD8(Ki-67)	2.65	8.14	< 0.0001	6.82	0.0011
IL-10	2.34	2.8	*0.0136	3.32	*0.0096
CD4(IL-10)	2.955	3.96	*0.0044	4.075	*0.0153
CD8(IL-10)	5.92	9.94	*0.0197	11.34	*0.0221
TGFβ	1.37	2.14	*0.0172	1.905	0.2822
CD3CD4(TGFβ)	1.08	2.165	*0.0441	1.94	0.3082
CD3CD8(TGFβ)	4	6.52	*0.0207	5.705	*0.0221
IFNγ	30.57	888.3	< 0.0001	279.5	< 0.0001
IL-6	90.25	12495	< 0.0001	2014	< 0.0001
IL-7	1.16	8.655	< 0.0001	3.325	0.0012
IL-10	3.355	26.12	< 0.0001	8.125	0.0015
IL-13	1.635	297	< 0.0001	3.24	< 0.0001
IL-17	1.065	15.61	< 0.0001	2.945	0.0037

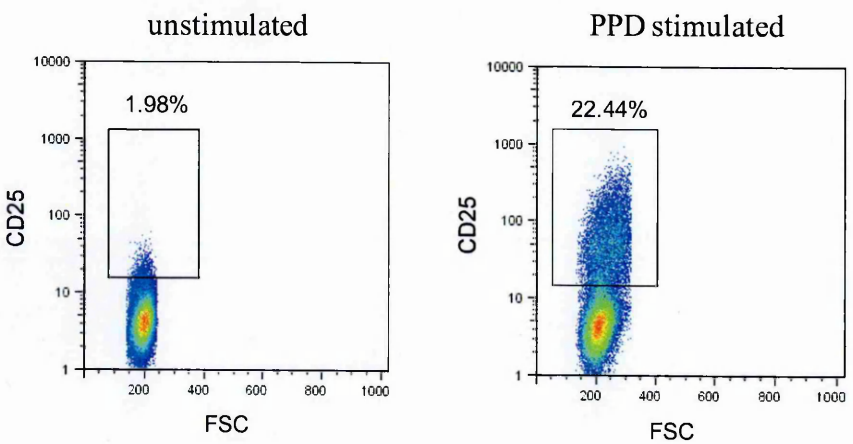
Blue text = significant upregulation compared to unstimulated at 5% significance
Red text = significant downregulation compared to unstimulated at 5% significance
* Not significant after Bonferroni correction

Table 5.1: In vitro responses in Group 1 (vaccinated at birth) at 4½ months. *500 µL whole blood diluted 1: 5 was cultured with PPD and BCG for 5 days. Table represents comparisons between T cell phenotypes (%) and cytokine production (pg/ml) from supernatants in unstimulated and stimulated cultures in Group 1(vaccinated at birth). A Wilcoxon non-parametric paired test was applied at 5% significance, n = 40 +/- 4.*

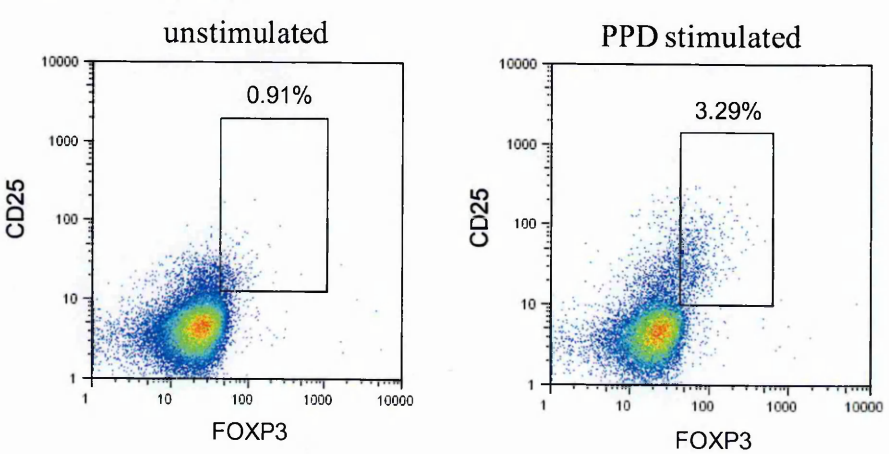
A unvaccinated



B BCG vaccinated



C unvaccinated



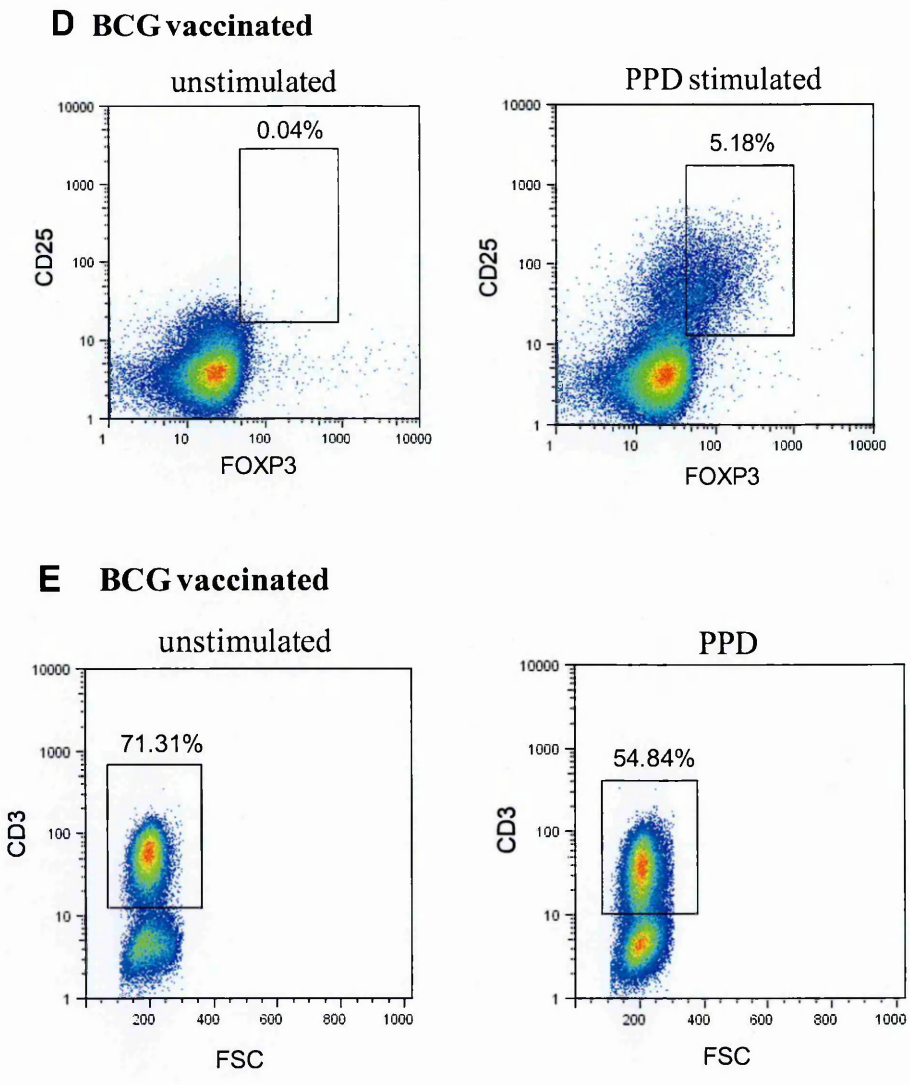


Figure 5.3: Flow cytometry plots of PPD stimulated whole blood. *500 µL whole blood diluted 1: 5 was cultured with PPD for 5 days. At 4½ months of age BCG vaccinated subjects (B, D and E) and unvaccinated (A and C) illustrating $CD25^+$ T cells gated on $CD4^+$ T cells (A and B) and $CD25^+FOXP3^+$ T cells gated on $CD4^+$ T cells (C and D) (unstimulated samples on the left and PPD stimulated samples on the right). (F) Downregulation of $CD3^+$ after PPD stimulation compared to unstimulated gated on the lymphocyte population.*

In response to PPD in the BCG vaccinated individuals, a mean of 23% of all $CD4^+$ T cells expressed the activation marker CD25 (6.5% in unstimulated) and 10.8% of $CD4^+$ T cells were of a $CD25^+FOXP3^+$ phenotype (1.3% in unstimulated control) (Figure 5.4A

and B). Interestingly the BCG naïve group also had increased activated T cells and Tregs in response to PPD stimulation *in vitro* at 4½ months (Figure 5.4A and B) but at significantly lower levels than the vaccinated group (Figure 5.4C and D).

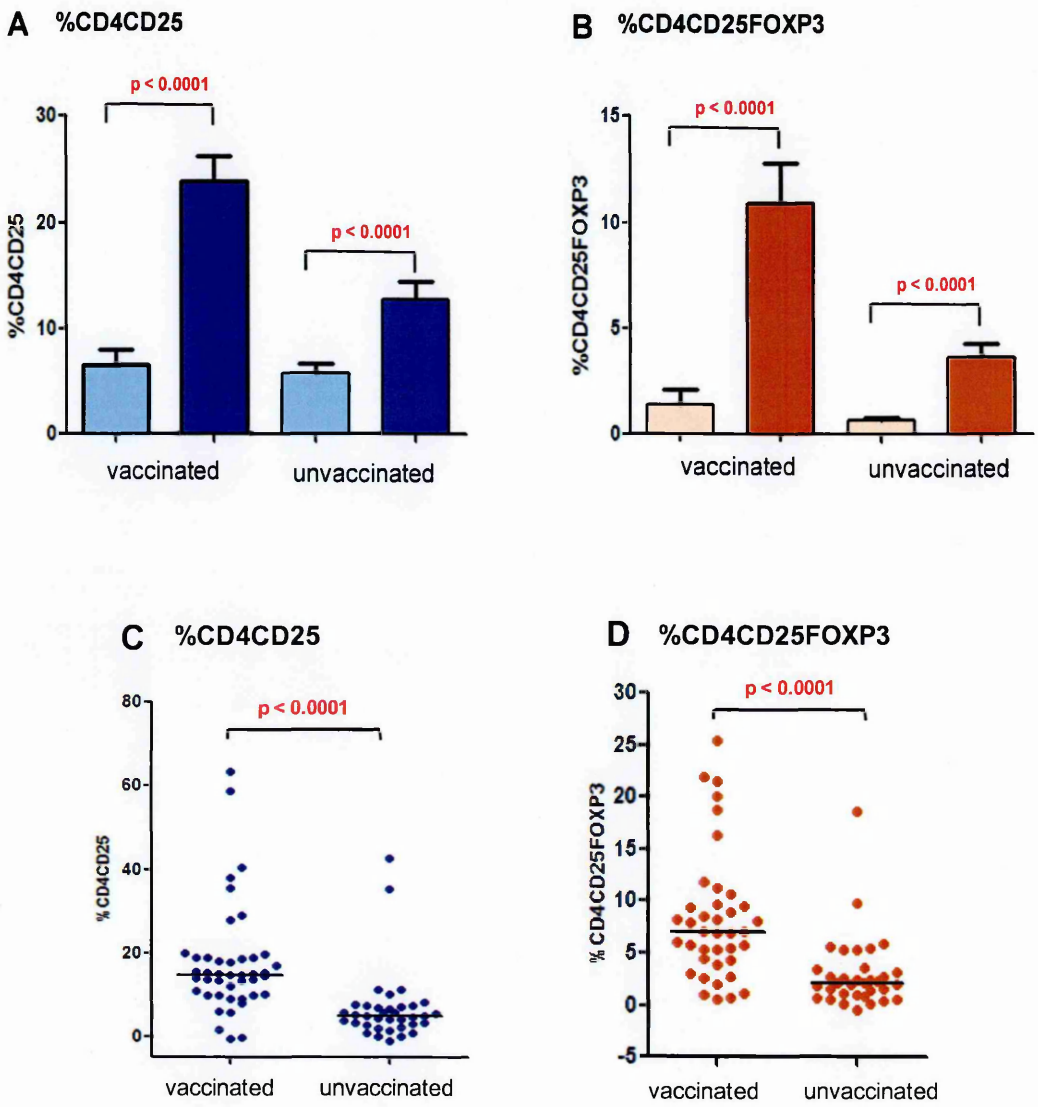


Figure 5.4: In vitro flow cytometry responses to PPD at 4½ months. *500 µL whole blood diluted 1: 5 was cultured with PPD for 5 days. Comparisons between unstimulated (pale bars) and stimulated (dark bars) cultures of $CD4^+CD25^+$ (A), $CD4^+CD25^+FOXP3^+$ (B), bars represent means and SEM error bars. A Wilcoxon non-parametric paired test was applied at 5% significance, Group 1 n = 43, Group 2 n = 38. Comparisons between Group*

*1 (vaccinated) and Group 2 (unvaccinated) after subtracting the background for $CD4^+CD25^+$ (C), $CD4^+CD25^+FOXP3^+$ (D), median values are presented as black line. A Mann Whitney U test was applied to compare the groups, Group 1 n = 40, Group 2 n = 36, *not significant after Bonferroni correction for multiple testing.*

In both groups, $CD4^+$ and $CD8^+$ T cells proliferated and produced IL-10 to PPD compared to the unstimulated controls although after adjustment for multiple testing this was not significant in the BCG vaccinated group (Figure 5.5). IL-10 production after subtracting the unstimulated values was comparable between groups suggesting that T cell production of IL-10 is due to NTM exposure rather than BCG vaccination. The evident production of IL-10 by T cells contrasted to cord blood responses to PPD where IL-10 was not T cell derived. Interestingly there was a trend for $CD8^+$ T cells to produce more IL-10 than $CD4^+$ T cells, particularly in unstimulated cultures which was consistent in all conditions studied and across both groups (Figure 5.5).

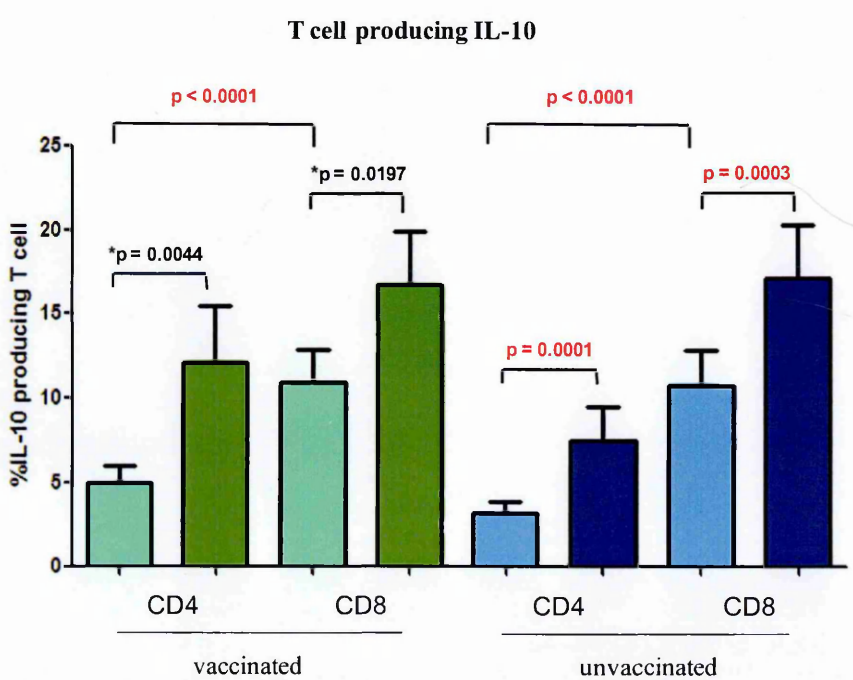


Figure 5.5: T cell derived IL-10 production in PPD stimulated cultures by flow

cytometry. *At 4½ months of age 500 µL whole blood diluted 1: 5 was cultured with PPD for 5 days. Comparisons between unstimulated (pale bars) and PPD stimulated (dark bars) for CD4 and CD8 T cell production of IL-10 and the difference between CD4 and CD8 production of IL-10 in the unstimulated cultures in Group 1 (vaccinated, green) and Group 2 (unvaccinated, blue), bars represent means and SEM error bars. A Wilcoxon non-parametric paired test was applied at 5% significance, Group 1 n = 42, Group 2 n = 37.*

In concordance with the activated response described above, all cytokines studied at 4½ months were upregulated in response to PPD in both the BCG vaccinated and unvaccinated groups including the Th1 cytokine IFNγ, the Th2 cytokines IL-13 and IL-6, the Th17 cytokine IL-17 and IL-10 (Figure 5.6A – C). IL-10 can be produced by a number of T cells including Th1, Th2 and Tr1 regulatory T cells in addition to non-T cell sources as mentioned previously (O'Garra and Vieira 2007). The upregulation of IL-10 in culture supernatants was similar in both groups (Figure 5.6E) further supporting the earlier suggestion that IL-10 is not induced in response to the BCG vaccine but is probably due to exposure to NTM. This is in contrast to IFNγ and IL-13 which was induced in BCG naïve individuals but at much lower concentrations than in the vaccinated group (Figure 5.6D and F). IL-17 was upregulated in response to PPD in both the vaccinated and unvaccinated groups although this was at a low concentration. A comparison between groups at 4½ months showed greater induction of IL-17 in the BCG vaccinated group (Group 1 median 14.55 pg/mL) compared to the unvaccinated group (Group 2 median 1.53 pg/mL, $p < 0.0001$, data not shown). Thymic function is controlled by IL-7 and neonatal naïve $CD4^+$ and $CD8^+$ T cells are explicitly responsive to IL-7 therefore production of this cytokine was studied. IL-7 was induced in both groups but at very low levels. A high level of the innate cytokine, IL-6 was induced in response to PPD in both groups but was higher in the vaccinated infants (median 11,923 pg/mL) compared to the unvaccinated group (median 1,213, $p < 0.0001$).

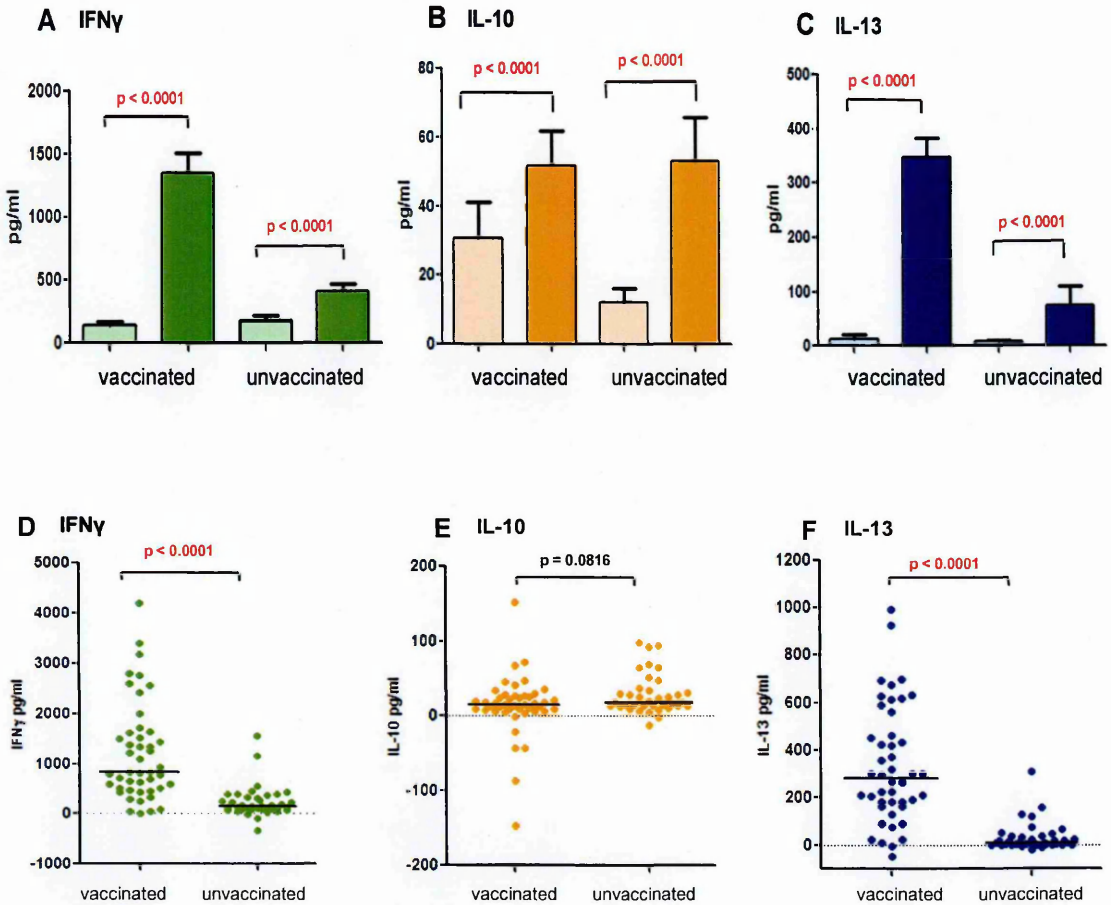


Figure 5.6: In vitro cytokine responses to PPD at 4½ months. *500 µL whole blood diluted 1: 5 was cultured with PPD for 5 days. Comparisons between unstimulated (pale bars) and stimulated (dark bars) cultures of IFNγ (A), IL-10 (B) and IL-13 (C), bars represent means and SEM error bars. A Wilcoxon non-parametric paired test was applied at 5% significance, Group 1 n = 46, Group 2 n = 39 for all cytokines. Comparisons between Group 1 (vaccinated) and Group 2 (unvaccinated) after subtracting the unstimulated background for IFNγ (D), IL-10 (E) and IL-13 (F), median values are presented as black bars. A Mann Whitney U test was applied at 5% significance to compare the groups.*

The BCG naïve group also responded *in vitro* to the BCG antigen compared to unstimulated controls even though the children were not vaccinated. Thus a number of

parameters were upregulated in this group including IFNγ (~ 4 fold less than with PPD), although this was not the case for $CD4^+CD25^+$ activated T cells. Increased TGFβ production to BCG was demonstrated *in vitro*, an effect not observed with PPD stimulation. However, after adjusting for multiple comparisons the difference seen in TGFβ was not sustained. Responses to BCG between groups behaved in a similar way to PPD reactivity in that the BCG vaccinated group had higher levels of $CD4^+CD25^+$, $CD4^+CD25^+FOXP3^+$, IFNγ and IL-13 but comparable levels of IL-10 production.

Since the children in the study were closely monitored for exposure to TB, it was not surprising to find low responses to EC at 4½- and 9- months of life. However there was significant reactivity to EC with an induction of $CD4^+CD25^+$ and $CD4^+CD25^+FOXP3^+$ T cells which was comparable between groups and thus unlikely to be related to BCG vaccination. Interestingly, at 4½ months an EC specific increase in IFNγ, IL-6 and IL-10 cytokine production, compared to background, was confined to the BCG naïve group (IFNγ $p = 0.0078$, IL-6 $p = 0.0013$, IL-10 $p = 0.0004$) (Figure 5.7A). However when comparing the two groups directly, based on subtracting the unstimulated control from the stimulated values, these differences were not apparent for IFNγ (Figure 5.7B and C) and IL-6 (Figure 5.7B), but IL-10 was still significantly higher in the unvaccinated group (Figure 5.7B and D) suggesting that BCG vaccination could bias away from an immunosuppressive IL-10 response and that there is some cross reactivity of ESAT-6/CFP-10 expressing NTM, such as *M. marinum* which has been shown to be present in The Gambia (Corrah 1994).

There were no differences in responses to EC between groups at 9 months of age or between groups 4½ months post BCG vaccination (i.e. Group 1 at 4½ months and Group 2 at 9 months).

A

	GROUP 1			GROUP 2		
	UNS	**ESAT-6/ CFP-10**	***p value***	**UNS**	**ESAT-6/ CFP-10**	***p value***
IFNγ	30.57	53.77	0.0738	67.43	68	0.0078
IL-6	90.25	132.1	0.1424	106.6	199.8	0.0013
IL-7	1.16	1.32	0.9678	1.32	1.32	0.3142
IL-10	3.355	4.305	0.8611	3.4	4.13	0.0004
IL-13	1.635	4.775	0.0535	3.21	7.18	0.071
IL-17	1.065	0.13	0.9569	0	0	0.7943

Blue text = significantly greater values after EC stimulation

B

	ESAT-6/ CFP-10 FP		
	Vaccinated (V)	**Unvaccinated (UV)**	***p value***
IFNγ	4.74	12.11	0.3915
IL-6	36.23	94.7	0.2919
IL-7	0	0	0.3572
IL-10	-0.19	1.29	0.0084
IL-13	0.23	0.76	0.8530
IL-17	0	0	0.6974

Red text = significantly lower values in BCG vaccinated group compared to unvaccinated

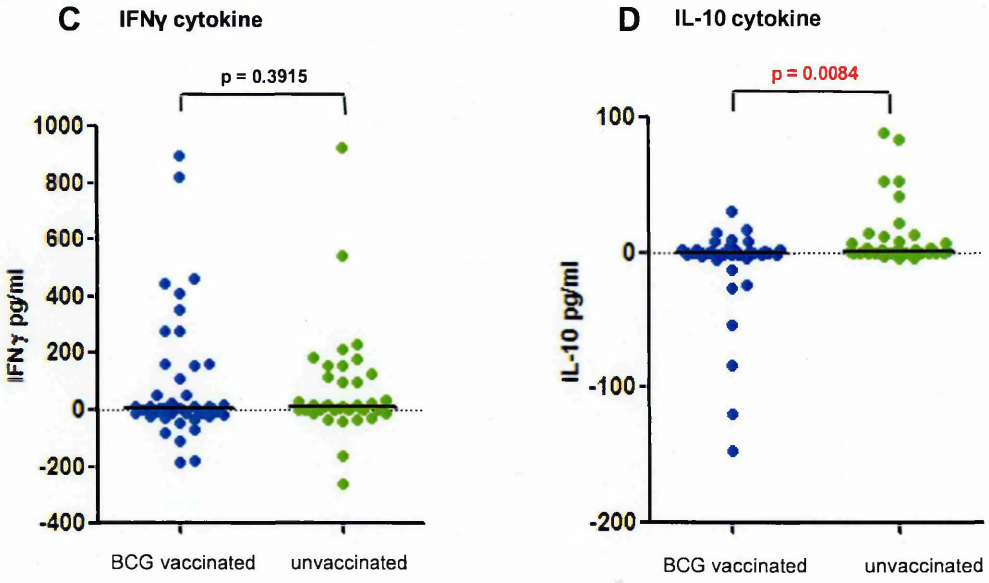


Figure 5.7: Cytokine responses to ESAT-6/ CFP-10 fusion protein at 4½ months by group. *500 µL whole blood diluted 1: 5 was cultured with EC for 5 days and cytokines were measured in the supernatants. (A) Table of cytokine responses (pg/ml) in unstimualted compared with EC stimulated cultures, a Wilcoxon non-parametric paired test was applied at 5% significance, Group 1 n = 46, Group 2 n = 39. (B) Table of comparisons of cytokine responses (pg/ml) between Group 1 (vaccinated) and Group 2 (unvaccinated) assessed by Mann Whitney U test. Comparison between Group 1 and Group 2 of IFNγ (C) and IL-10 (D) cytokine production after subtracting unstimulated control in response to EC.*

Since responses to PPD were observed at birth it was important to compare these responses to those observed at 4½ months in the unvaccinated group in order to determine how much reactivity might be attributable to exposure to NTM over the first 4½ months of life. Surprisingly, a number of the responses observed at 4½ months to PPD, BCG and EC were similar to those observed at birth (Table 5.2). These included T cell proliferation and IL-10 production by T cells and within the culture supernatant. There was a trend for IL-10 cytokine production (CD8+IL-10+ and IL-10 from supernatants) to be increased at 4½ months but this was not significant after correction for multiple testing. However,

mycobacterial induced activated T cells and Tregs, IFNγ and IL-13 responses were all higher at 4½ months compared to birth suggesting either there is some NTM exposure at birth that influences the T cell proliferation and IL-10 production in response to PPD or the responses observed at 4½ months were non-specific and too low to regard as true responses. In the BCG vaccinated group all responses at 4½ months of age were upregulated compared to birth (data not shown).

	At birth	4½ months	*p value*
CD3	-1.69	-5.505	0.1101
CD4	-0.8	-5.55	0.0727
CD8	0.76	0.51	0.4441
CD4(CD25)	0.87	4.93	0.0004
CD4(CD25FOXP3)	0.21	4.77	< 0.0001
Ki-67	1.43	0.53	0.5115
CD4(Ki-67)	1.4	0.94	0.4624
CD8(Ki-67)	2.82	1.08	0.6879
IL-10	0.51	0.87	0.1925
CD4(IL-10)	0.7	1.6	0.1554
CD8(IL-10)	1.01	1.54	*0.0304
TGFβ	0.77	-0.015	0.9713
CD3CD4(TGFβ)	0.59	0.04	0.5401
CD3CD8(TGFβ)	0.98	0.045	0.6567
IFNγ	0	142.1	< 0.0001
IL-10	8.13	19.56	*0.0414
IL-13	0	9.26	< 0.0001

Blue text = significant upregulation at 4½ months compared to at birth
* Not significant after Bonferroni correction

Table 5.2: In vitro responses to PPD at birth and at 4½ months in Group 2. *500 µL whole blood diluted 1: 5 was cultured with PPD for 5 days. Table represents T cell phenotypes (%) and cytokine production (pg/ml) at birth compared to 4½ months of age in Group 2 (vaccinated at 4½ months of age). Values presented are median values in response to antigen after subtracting the unstimulated control. A Wilcoxon non-parametric paired test was applied at 5% significance, n = 30.*

5.2.3 Comparison of mycobacterial responses after different BCG vaccination schedules

Since the two groups received BCG vaccination at different time points it was important to compare responses between groups in two ways. First, a comparison of responses at 9 months of age i.e. 9 months post vaccine in Group 1 and 4½ months post vaccine in Group 2. Secondly, reactivity 4½ months after BCG vaccination i.e. 4½ months of age in Group 1 and 9 months of age in Group 2.

Despite the clear differences described between groups in reactivity at 4½ months of age, it was clear that by 9 months of age measured responses to mycobacterial antigens were similar in both groups (Table 5.3). This suggests that delaying the BCG vaccine to 4½ months did not affect mycobacterial specific immunity at 9 months of age.

	Group 1 (vacc. at birth)	**Group 2 (vacc. at 4½ m)**	*p value*
CD3	-5.56	-10.84	*0.0468
CD4	-4.51	-10.03	*0.0085
CD8	0.85	1.345	0.3661
CD4(CD25)	11.43	14.01	0.2099
CD4(CD25FOXP3)	4.87	6.215	0.3475
Ki-67	4.25	5.05	0.4603
CD4(Ki-67)	5.97	6.57	0.7594
CD8(Ki-67)	4.22	7.355	0.1844
IL-10	1.07	2.935	0.0765
CD4(IL-10)	1.17	3.72	0.3939
CD8(IL-10)	2	5.115	0.3372
TGFβ	0.34	1.02	0.2630
CD3CD4(TGFβ)	0.46	0.9	0.3747
CD3CD8(TGFβ)	2.78	2.32	0.4933
IFNγ	254.0	214.8	0.9544
IL-10	20.70	13.65	0.2064
IL-13	287.9	374.4	0.5191

* Not significant after Bonferroni correction

Table 5.3: Comparing in vitro responses to PPD at 9 months of age between groups. *500 µL whole blood diluted 1: 5 was cultured with PPD for 5 days. Table represents*

*comparisons of T cell phenotypes (%) and cytokine production (pg/ml) between Group 1 and Group 2 at 9 months of age. A Mann Whitney test was applied at 5% significance, Group 1 n = 43, Group 2 n =38 . * Not significant after Bonferroni correction.*

When comparing the two groups 4½ months post BCG vaccine most parameters were also similar. However IFNγ production was lower in Group 2 (vaccinated at 4½ months) compared to Group 1 (vaccinated at birth) (Figure 5.8A). IL-13 production was similarly upregulated in both groups (Figure 5.8B). IL-10 reactivity in response to PPD was similar 4½ months after vaccine, however the BCG antigen induced IL-10 responses that were higher in the delayed vaccine group (Figure 5.8C and D).

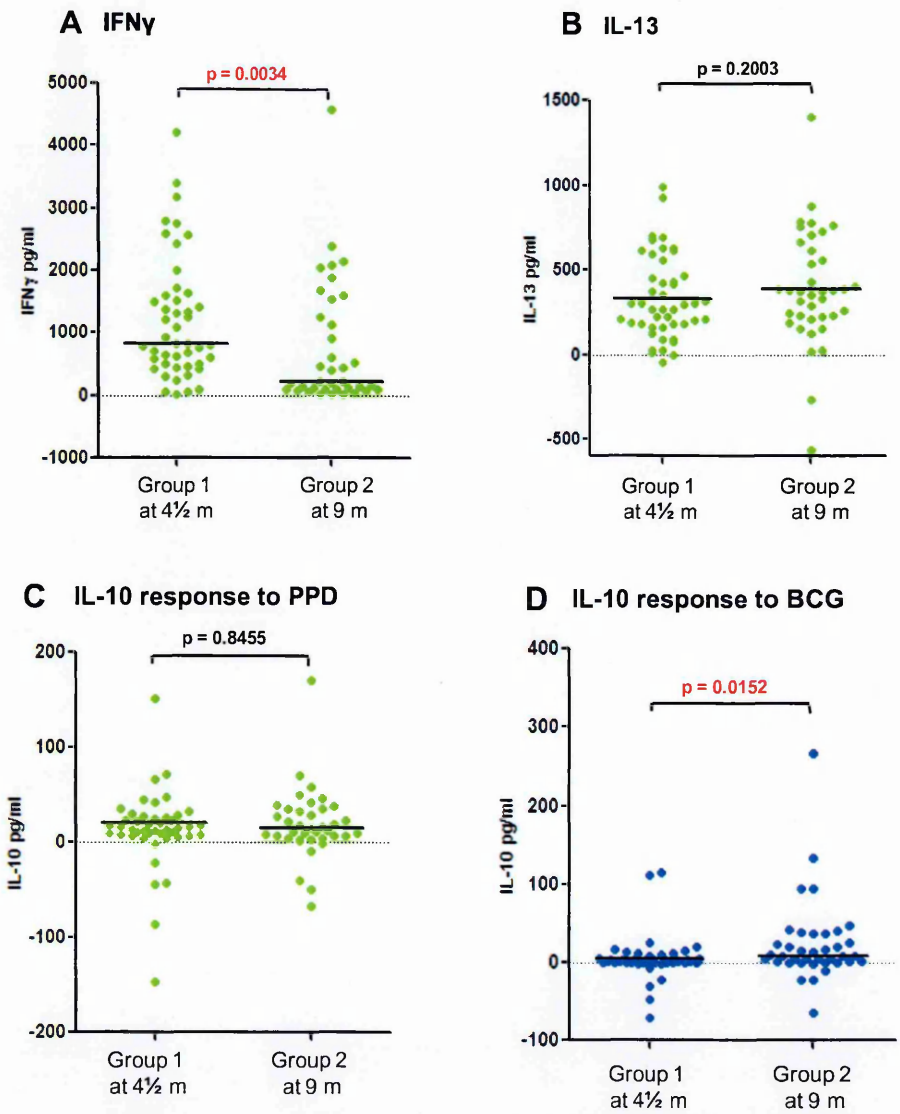


Figure 5.8: Responses to PPD antigen 4½ months after BCG vaccine. *500 µL whole blood diluted 1: 5 was cultured with PPD and BCG for 5 days. IFNγ (A), IL-13 (B), IL-10 (C) production in response to PPD and IL-10 production in response to BCG (D). Comparisons between Group 1 at 4½ months of age and Group 2 at 9 months of age after subtracting the background, median values are presented as black bars. A Mann Whitney U test was applied to compare the groups, Group 1 n = 46, Group 2 n = 37.*

Despite the lower IFNγ production 4½ months post vaccination in Group 2 in response to PPD, both IFNγ and IL-13 were still increased compared to pre-vaccine responses at 4½ months of age demonstrating that a mixed Th1, Th2 response is induced in the delayed vaccine group (Figure 5.9A and B). However, IFNγ levels induced in the delayed BCG vaccine group were lower than those observed when vaccinating at birth. It is evident however, that there were some strong IFNγ responders post-BCG vaccine although most are poor responders (Figure 5.9A).

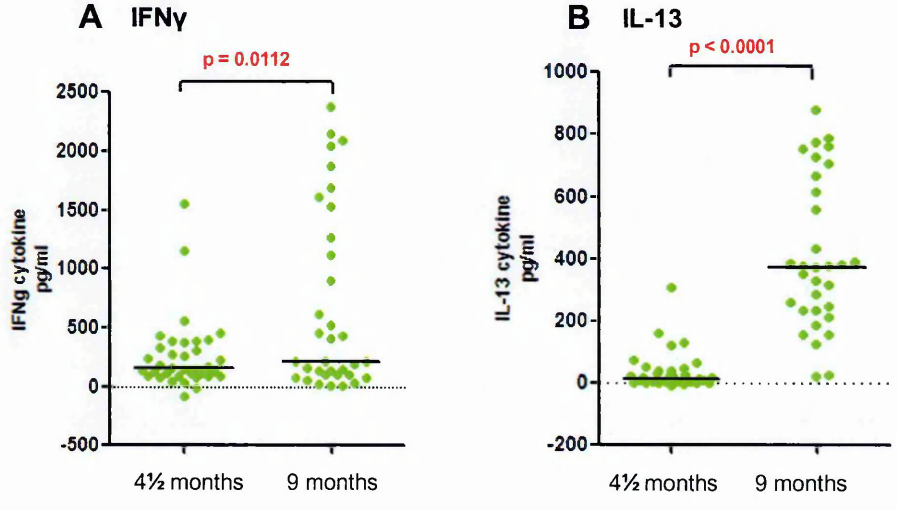


Figure 5.9: Cytokine production over time in Group 2. *500 µL whole blood diluted 1: 5 was cultured with PPD for 5 days. IFNγ (A) and IL-13 (B) production in response to PPD were compared over time in Group 2 (vaccinated at 4½ months) after subtracting the*

background, median values are presented as black bars. A Wilcoxon non-parametric paired test was applied at 5% significance, Group 1 n = 46, Group 2 n = 39.

As part of the original hypothesis it was suggested that by 4½ months the infants will have been exposed to NTM and this may impair the IFNγ response to BCG vaccine at 9 months of age. Therefore, infants that responded well to PPD at 4½ months in the unvaccinated group might be expected to have a reduced response to PPD or BCG post vaccine (at 9 months of age). A scatter plot of correlation between PPD IFNγ responses at 4½ months compared to PPD IFNγ responses at 9 months in Group 2 (Figure 5.10A), showed no correlation (Spearman r = 0.07568 p = 0.6609). Indeed most values were low at both time points, although a subgroup of 11 of the 36 individuals had increased IFNγ production at 9 months (> 1,000 pg/ml), thus a good response to the BCG vaccine, although they did not correspond to a low IFNγ group at 4½ months. Correlations with BCG responses illustrated similar findings.

It was also hypothesised that Tregs may be responsible for the attenuated response found in Group 2, however there was no correlation between $FOXP3^{+}$Tregs at 4½ months and IFNγ responses at 9 months suggesting that the reduced IFNγ observed at 9 months in the delayed vaccine group was not a result of PPD stimulated Tregs present at 4½ months of age (Figure 5.10B). There was also no association between IL-10 cytokine production at 4½ months in Group 2 and IFNγ production at 9 months (Figure 5.10C), suggesting that this cytokine is not responsible for the lower IFNγ responses.

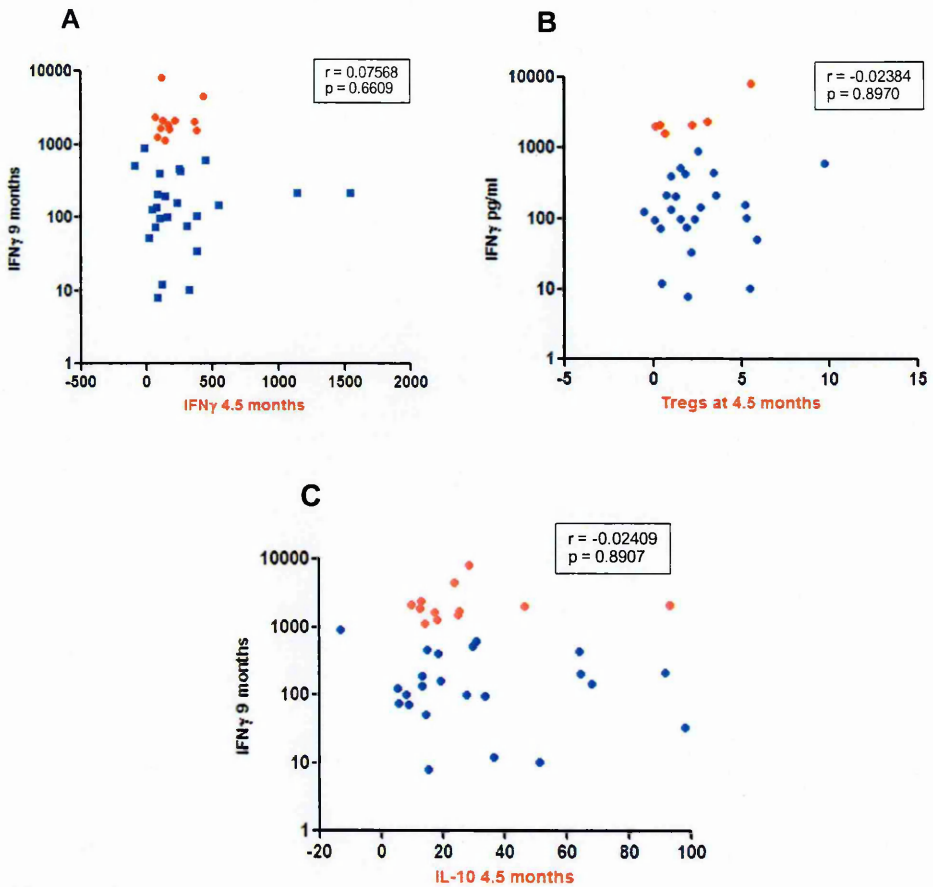


Figure 5.10: Scatter plots of comparisons between IFNγ responses to PPD at 9 months to immune correlates at 4½ months of age in Group 2. *500 µL whole blood diluted 1: 5 was cultured with PPD for 5 days. The relationship between cytokine responses to PPD at 9 months (4½ months after delayed BCG vaccine) to pre-vaccination baseline at 4½ months of age for IFNγ (A), FOXP3^{+}Tregs (B) and IL-10 (C) was assessed after subtracting the background. Spearman's correlation coefficient suggested there was no correlation between responses at 4½- to 9- months of age (p = 0.6609, 0.8970, 0.8907 respectively), n = 36. Red dots represent 11 individuals that responded well (> 920 pg/mL) to PPD at 9 months of age.*

5.2.4 PPD responses waned 9 months after BCG vaccination

An important question both clinically and immunologically is how long does the

strong Th1 memory immune response remain in those vaccinated at birth. Comparing responses in group 1 (vaccinated at birth) at 4½- and 9- months of age may help answer this question. No responses measured were increased in response to PPD over this time point. In the majority of cases the responses were similar suggesting persistence for 9 months, however some key immune responses were downregulated.

The percentage of $FOXP3^{+}$Tregs in response to PPD had decreased by 9 months of age although the percentage of $CD4^{+}CD25^{+}$ cells remained stable. IFNγ (the cytokine thought to play a major protective role against *M.tb)* production in response to PPD was downregulated at 9 months compared to 4½ months (medians of 920.3 and 254 pg/mL respectively) (Figure 5.11A) without a simultaneous downregulation of IL-10 or IL-13, suggesting that the Th1 response to vaccine has reduced by 9 months but the Th2 and regulatory responses persisted. There was an overall positive correlation between IFNγ responses to PPD at 4½- and 9- months of age ($r = 0.4313$, $p = 0.0039$) suggesting those that responded well to PPD at 4½ months of age were more likely to be good responders at 9 months of age although the predictive r value was low (Figure 5.11B). Nine subjects had an IFNγ response, greater than 920 pg/ml (median value at 4½ months) at 9 months of age (Figure 5.11A). When examining for an association with the IFNγ responses in these individuals at 4½ months, there was no correlation ($r = 0.08333$, $p = 0.8432$) suggesting the correlation observed previously was not due to the higher responders.

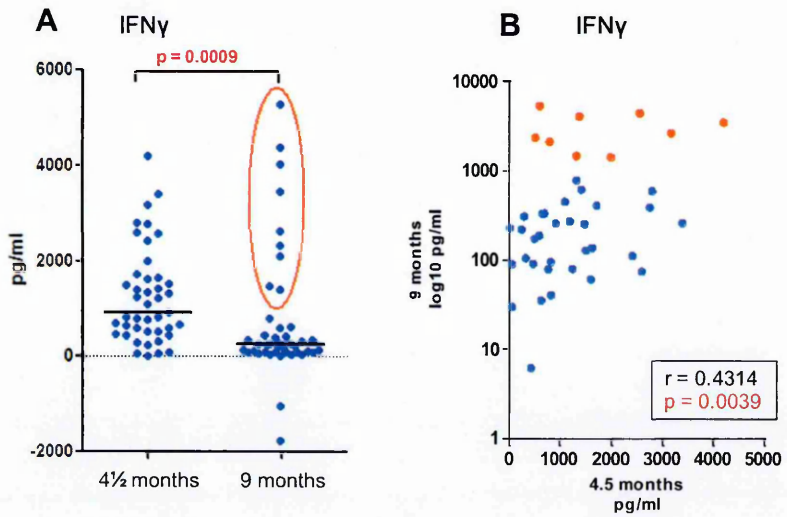


Figure 5.11: Longitudinal responses within Group 1 comparing 4½ months post vaccine to 9 months post BCG vaccine in response to PPD. *500 μL whole blood diluted 1: 5 was cultured with PPD for 5 days. IFNγ responses to PPD 4½- and 9- months after BCG vaccination in Group 1 (A) and the relationship between responses in the same individuals over time (B) was assessed after subtracting the background. Red dots and red oval represent 9 individuals that responded well (>920 pg/mL) to the PPD at 9 months of age in Group 2. Longitudinal data was compared using a Wilcoxon non-parametric paired test at 5% significance, n = 43. Spearman's correlation coefficient measured the correlation between responses at 4½- and -9 months of age, n = 43.*

Table 5.4 illustrates the profile of immune responses to PPD across the three time points for Group 1 (vaccinated with BCG at birth). Clearly different patterns emerged for the different populations of cells analysed. At 4½ most of the subjects exhibited an increase in activated T cells in those vaccinated at birth, approximately half of these then had responses that reduced at 9 months while half had responses that increased, illustrating heterogeneity in the responses. In contrast, the frequency of $CD4^+CD25^+FOXP3^+$ Tregs increased at 4½ months but in the majority of subjects the percentage was reduced by 9 months. Thus at 9 months the responses were more activated and less suppressive, but this did not correspond to the IFNγ production that was predominantly reduced at 9 months. By 4½ months of age, all individuals upregulated IFNγ in response to PPD compared to that observed in cord blood, but 82% of individuals had a reduced response by 9 months. IL-13 fitted a similar profile to the activated T cells where most subjects exhibited an increase in IL-13 in response to PPD at 4½ months, but half of those had a sustained increase in IL-13 and half had a reduction in IL-13 by 9 months. IL-10 was the only parameter that, at 4½ months of age, had approximately equal numbers of subjects with an increase or a decrease in IL-10 reactivity.

	months	 0 4½ 9	 0 4½ 9	 0 4½ 9	 0 4½ 9
Group 1		**DEC DEC (%)**	**DEC INC (%)**	**INC DEC (%)**	**INC INC (%)**
CD4		10 (31.3)	14 (43.8)	7 (21.9)	1 (3.1)
CD8		6 (30)	5 (25)	7 (35)	2 (10)
CD4(CD25)		1 (3.1)	1 (3.1)	16 (50)	14 (43.8)
CD4(CD25FOXP3)		3 (9.4)	8 (25)	18 (56.3)	3 (9.4)
IFNγ		0	0	31 (81.6)	7 (18.4)
IL-10		6 (15.8)	12 (31.6)	9 (23.7)	11 (29.0)
IL-13		0	2 (5.3)	20 (52.6)	16 (42.1)

Table 5.4: Immune response profile to PPD in Group 1 that were vaccinated at birth. *Comparing profiles of responses over time from birth to 4½ months and from 4½ months to 9 months in Group 1 that were vaccinated with BCG at birth. Numbers and percentages (in brackets) of individuals that had immune responses more or less than previous time points were calculated, DEC = decrease-, INC = increase- in responses to PPD compared to earlier time point. Therefore DEC DEC is interpreted as numbers of subjects that had a decrease from birth to 4½ months AND a decrease from 4½ months to 9 months.*

Figure 5.12A – E summaries the overall longitudinal responses to PPD for activated T cells, $FOXP3^{+}$Tregs, IFNγ, IL-10 and IL-13 cytokine production between all three time points in Group 1 and 2 that have been discussed in detail throughout this chapter.

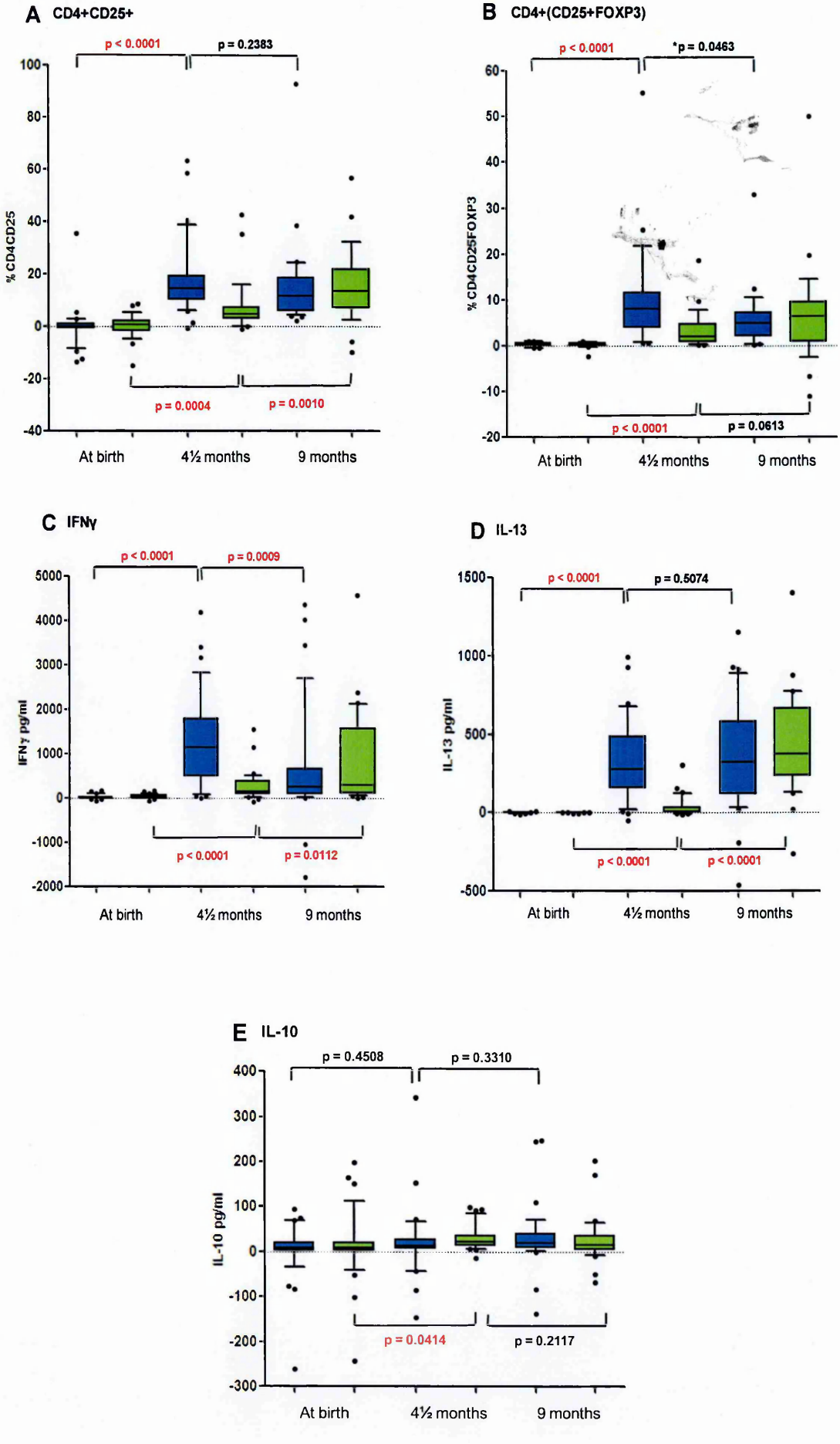

A CD4+CD25+
p < 0.0001
p = 0.2383
% CD4CD25
p = 0.0004
p = 0.0010
At birth
4½ months
9 months
B CD4+(CD25+FOXP3)
p < 0.0001
*p = 0.0463
% CD4CD25FOXP3
p < 0.0001
p = 0.0613
At birth
4½ months
9 months
C IFNγ
p < 0.0001
p = 0.0009
IFNγ pg/ml
p < 0.0001
p = 0.0112
At birth
4½ months
9 months
D IL-13
p < 0.0001
p = 0.5074
IL-13 pg/ml
p < 0.0001
p < 0.0001
At birth
4½ months
9 months
E IL-10
p = 0.4508
p = 0.3310
IL-10 pg/ml
p = 0.0414
p = 0.2117
At birth
4½ months
9 months

Figure 5.12: Longitudinal responses to PPD over time. *Longitudinal $CD4^+CD25^+$ (A), $CD4^+CD25^+FOXP3^+$ (B), IFNγ (C), IL-13 (D) and IL-10 (E) responses to PPD after 5 days of culture in Group 1 (blue) and Group 2 (green). Unstimulated values were subtracted from stimulated values. Black bar represents the median value of the data. Wilcoxon paired non-parametric test was applied at 5% significance.*

5.3 DISCUSSION

Young children in The Gambia receive numerous vaccines before the age of 9 months including BCG, HBV, DTwP, Hib, OPV, measles (MV) and yellow fever (YF). BCG is given at birth or at first contact with the child but immunological evidence is limited with respect to optimal timing of BCG in infants. Many studies have shown that BCG is less effective in developing countries and this study assessed the immunological effects of giving BCG vaccine at two different time points to analyse the impact of exposure to NTM prior to vaccination.

5.3.1 Mycobacterial responses in cord blood

The findings of *in vitro* recall responses to mycobacterial antigens in cord blood were perhaps unexpected. Increased T cell proliferation and induction of IFNγ was observed in response to PPD alongside an increased regulatory response of $CD4^+CD25^+FOXP3^+$ Tregs and induced IL-10 (although not from T cells). Previous studies have found that mycobacterial responses in cord blood were due to a lipid component of mycobacterial lysate. However, in contrast to our results, this study found no responses to PPD. The authors found an expansion of gamma delta (γδ) T cells and proposed that when these T cells in a newborn are confronted by a mycobacterium, they respond non-specifically to the lipid component of the organism (Tsuyuguchi, Kawasumi et al. 1991). Our results showed similar responses to BCG supporting the idea that a non-specific component in the mycobacterial antigens used in our study acts as an immunostimulatory antigen, however responses to the specific ESAT-6/CFP-10 fusion protein would not support this as it does not contain other mycobacterial components.

Limited evidence suggests that cell-mediated immunity could be transferred from mother to foetus *in utero* or through breast milk. Earlier studies have demonstrated this by leukocyte migration inhibition assays, where induction of leukocyte inhibitory factor (LIF) in antigen stimulated cultures inhibits lymphocyte migration on agar, and

lymphoproliferative assays using radiolabelled thymidine (Gallagher, Welliver et al. 1981; Pabst, Godel et al. 1987). Interestingly these studies showed a stimulation index (SI) of > 2.0 in cord blood cultured with PPD that decreased to 1- 2 by 6 months of age which correlated to a reduction in migration inhibition in the LIF assay and to PPD responses from the mother. It was thought that maternal antibodies that are known to cross the placenta contribute to the cell mediated immunity and suggest that specific factors (e.g. Transfer factor) may be responsible for this (Pabst, Godel et al. 1987). Several studies have found T cell populations present in breast milk (Schlesinger and Covelli 1977; Girdhar, Girdhar et al. 1981; Sabbaj, Ghosh et al. 2005) although this would not contribute to the findings in cord blood prior to being breast fed. Foreign antigen exposure *in utero* was originally thought to induce immunological tolerance (Billingham, Brent et al. 1953), but many studies have found that transfer of foreign proteins, parasite antigens and even cells from mother to the foetus can induce memory pro-inflammatory immune responses although the mechanisms involved remain unclear (Gitlin, Kumate et al. 1964; King, Malhotra et al. 2002; Adams and Nelson 2004; Malhotra, Mungai et al. 2006) (Malhotra, Ouma et al. 1997; Malhotra, Mungai et al. 1999; Brustoski, Moller et al. 2006). Active placental malaria at delivery was associated with increased frequencies of Plasmodium falciparum (Pf)-specific $CD4^{+}IL\text{-}10^{+}$ T cells and *ex vivo* $CD4^{+}CD25^{+}CTLA\text{-}4^{+}$ Tregs in cord blood (Brustoski, K *et al*, 2005, Brutoski, K *et al*, 2006) which is in agreement with the increased $FOXP3^{+}$Tregs that were observed in response to PPD in the cord blood of our participants. Malhotra *et al* also showed that cord blood from 30% of Kenyan neonates induced IFNγ production after PPD stimulation but with no Th2 cytokines induced. In contrast none of the US neonates responded to PPD (Malhoatra, I *et al*, 1997). Interestingly Ben-Smith *et al* compared T cell phenotypes between Malawi and the UK and found that although Malawian adolescents had a lower percentage of naïve, and a higher percentage of memory T cells, cord blood T cell populations were similar in both countries (Ben-Smith, Gorak-Stolinska et al. 2008). Many studies examining allergen sensitivity in the

neonate have found allergen-responsive T cells in cord blood but these lacked the fine specificity of adult memory T cells and were often not $CD45RO^+$ T cells (Yabuhara, Macaubas et al. 1997; Thornton, Upham et al. 2004). Interestingly many animal studies have observed *in utero* transmission of *M. avium* subspecies *paratuberculosis* (MAP) (Sweeney, Whitlock et al. 1992; Judge, Kyriazakis et al. 2006; Alinovi, Wu et al. 2009; Singh, Sohal et al. 2009). In cattle MAP was isolated from the placenta, foetus and colostrum of infected mothers suggesting vertical transmission of mycobacteria (Sweeney, Whitlock et al. 1992; Sweeney, Whitlock et al. 1992). In addition, in 1 day old unvaccinated cattle neonates, $CD8^+CD3^-$ NK-like cells produce IFNγ in response to BCG infected DCs (Hope, Sopp et al. 2002). It is possible that the IFNγ that was observed in our studies was produced from NK cells but we could not confirm this.

It is important to recognise the possibility that mature immune responses might be due to maternal contamination of cord blood. This could occur *in utero* (Srivatsa, Srivatsa et al. 2003), during labour when the endothelial vessels of the umbilical cord become 'leaky' (Masuzaki, Miura et al. 2004) or during blood collection. In some immunodeficient circumstances, maternal cells cross the placenta and engraft into human foetal tissues *in utero*, resulting in 'maternal microchimerism'. A recent study identified human maternal cells residing in foetal lymph nodes that induced the development of $CD4^+CD25^{high}FOXP3^+$ Tregs that suppressed foetal anti-maternal immunity, and persisted until early childhood (Mold, Michaelsson et al. 2008). The contribution of maternal cells was not examined further in this study but HLA typing cellular samples from the mother and baby could identify the extent of this problem.

Another possibility that has been well studied in mice, is based on the promiscuous low affinity TCR/MHC peptide interactions that occur on the surface of functionally immature T cells (Le Campion, Lucas et al. 2002). It has been suggested that in neonatal mice this process may be mediated by a subset of T cells with CDR3 regions that are shorter than on adult TCRs which interact with the alpha chain of the MHC

molecule rather than using the peptide/peptide-binding groove (Gavin and Bevan 1995). T cell epitope mapping of responses to ovalbumin (OVA) in human cord blood indicated reactivity to multiple regions as opposed to an average of < 1 site when peripheral blood from 5 year olds was tested (Yabuhara, Macaubas et al. 1997).

The increase in IL-10 production observed in cord blood did not appear to arise from $CD4^+$ or $CD8^+$ T cells since by flow cytometry the upregulation of IL-10 was in total IL-10 producing cells, but not $CD4^+$ or $CD8^+$ T cells specifically. Many other cells produce IL-10 including macrophages (Watkins, Semple et al. 2008) and B cells (Sun, Deriaud et al. 2005) (O'Garra and Vieira 2007)) but these were not examined in this study. Watkins *et al* found that cord blood responded to BCG antigen with an induction of IL-10 from $CD14^+$ monocytes, accompanied by high IFNγ and moderate IL-13 production from T cells (Watkins, Semple et al. 2008). Although the amount of IL-10 produced in cord blood was low, DCs have been shown to be extremely sensitive to the inhibitory effects of small amounts of IL-10 (Mosser and Zhang 2008). It is possible that the IL-10 production observed in cord blood contributes to the defective function of antigen presenting cells found in neonates.

In Chapter 4 it was suggested that $CD4^+$ T cells were more susceptible to apoptosis. Cord blood responses to PPD showed increased $CD4^+$ proliferation and increased IFNγ and IL-10 production. It was interesting that both the proportion of $CD4^+$ T cells and the proportion of $CD4^+CD25^+$ T cells did not increase, but the proportion of $CD4^+CD25^+FOXP3^+$ T cells did. It is possible that there is an increase in $CD4^+$ and $CD4^+CD25^+$ T cells but these are more susceptible to apoptosis as was suggested in Chapter 4. In contrast, the $CD4^+$ T cells that express FOXP3 could be a more resistant subset of $CD4^+$ T cells and therefore were increased in response to PPD. This finding is in conflict with other studies that suggest Tregs are more susceptible to apoptosis (Fritzsching, Oberle et al. 2006; Vukmanovic-Stejic, Zhang et al. 2006). Interestingly Fritzsching *et al* showed that neonatal Tregs possessed a naïve phenotype but were more

resistant to CD95L-mediated apoptosis than adult Tregs which supports our finding. The use of apoptotic markers would confirm this idea and ascertain if $FOXP3^{+}$Tregs in cord blood are more or less susceptible to apoptosis.

5.3.2 Mycobacterial responses in infants with respect to the timing of BCG vaccination

In vitro responses to BCG and PPD were assessed in this study. Since the BCG vaccine is a live replicating bacteria, it is likely that processed antigens are presented on both MHC class I and II molecules stimulating both a CD4 and CD8 T cell response, whereas PPD, a mix of secreted antigens from *M.tb*, are likely to be loaded onto MHC class II molecules and induce a predominant CD4 response. However, most responses observed were similar between BCG and PPD which is most likely due to the cross reactivity between the immunogenic components of these mycobacterial antigens. RNA expression profiles of overnight responses to BCG and PPD in BCG vaccinated infants support this finding (Fletcher, Keyser et al. 2009).

Overall the timing of BCG vaccination affected the immune response to PPD in the short term but by 9 months of age there was no effect on the parameters tested. As reported in the literature, BCG at birth induced a Th1 IFNγ response to PPD (Marchant, Goetghebuer et al. 1999; Vekemans, Amedei et al. 2001; Ota, Vekemans et al. 2002) (Sander, Skansen-Saphir et al. 1995). Those vaccinated with BCG at 4½ months had much less IFNγ production to PPD but IL-13 levels were similar to the group vaccinated at birth. This was supported by Marchant, A *et al* where delaying BCG until 2 months of age resulted in a trend for reduced IFNγ responses 2 months later. It was also reported that the unvaccinated group showed no IFNγ responses but slightly higher IL-4 responses, although they did not look at IL-13. The lack of IFNγ response may be due to the sensitivity of the assays. Our study utilised the CBA system which is more sensitive than ELISAs and has been shown to produce results slightly higher in range than other systems (Elshal and McCoy 2006). By 12 months of age the levels of IFNγ were similar for all the groups but

were 10-fold lower than 2 months post vaccination (Marchant, Goetghebuer et al. 1999). Our study supports these findings and suggests that the IFNγ memory response is transient; waning by 9 months of age while the Th2 response is strong and persists longer and also supports the widely accepted paradigm that there is a Th2 bias to priming in early life and BCG priming is no exception. The loss of the Th1 response but maintenance of the Th2 response may be due to the increased susceptibility of Th1 cells to death after re-stimulation with antigen. In mice experiments, TCR-transgenic $CD4^+$ cells from neonates and adults were transferred into a neonatal host and found that the neonatal Th1 cells underwent apoptosis in response to re-exposure to antigen, whereby the Th2 cells remained unaffected (Li, Lee et al. 2004). This process is thought to be due to FAP-1, an inhibitor of Fas-related apoptosis which is present in Th2 cells but not Th1 cells (Zhang, Brunner et al. 1997). This may also explain the reduced IFNγ response to BCG vaccination in the delayed vaccine group after exposure to NTM as the primary stimulus.

Correlates of protection against TB are not well defined although IFNγ is a key regulator. However studies in mice have shown protection from TB without the need for IFNγ (Elias, Akuffo et al. 2005). Goldsack and Kirman propose that that IFNγ-mediated protection is required in the primary response rather than the secondary recall response and suggest Th17 and Tregs may be involved in overall protection independent of IFNγ (Goldsack and Kirman 2007). There are mice studies that support the idea that IFNγ is not the sole cytokine required for protection. BCG vaccination induced IFNγ prior to *M.tb* challenge but the vaccinated and unvaccinated mice induced similar levels of IFNγ post challenge even though BCG vaccinated mice exhibited protection (Elias, Akuffo et al. 2005). In addition BCG vaccinated $IFN\gamma^{-/-}$ mice were capable of reducing bacterial burden when challenged with *M.tb* (Cowley and Elkins 2003). This is interesting in light of our results where delaying the vaccine reduced the IFNγ response compared to BCG vaccination at birth. This may not have any relation to protective levels in these infants.

Previous studies have shown that IFNγ and IL-10 dynamics in response to PPD

after BCG vaccination were quite different with IFNγ production peaking at 8 weeks post vaccination and then declining by 12 months. IL-10 reactivity peaked at 2 weeks and declined to its lowest levels peak at 8 weeks i.e. a much earlier intervention than IFNγ (Nabeshima, Murata et al. 2005). We may therefore have missed the BCG induced IL-10 response when examining responses 4½ months after vaccination. These dynamics are also consistent with the waning of IFNγ responses by 9 months of age observed in our study.

We hypothesised that exposure to NTM might attenuate the immunogenicity of BCG. Our results showed that by 4½ months of age there were activated responses to PPD in the unvaccinated group which suggests mycobacterial priming. The lower IFNγ production observed in this group post vaccination compared to those vaccinated at birth would support this hypothesis. In addition we hypothesised that the attenuation of BCG would be associated with induction of Tregs. $CD4^+$ Tregs were defined by FOXP3 expression as a marker of natural Tregs, and by intracellular IL-10 as an indicator of Tr1 induced Tregs. Induction of $CD4^+CD25^+FOXP3^+$ T cells in response to PPD was observed in both groups, but to a greater extent in the vaccinated group. By contrast IL-10 upregulation (from $CD4^+$ and $CD8^+$ T cells) was observed in similar concentrations in the unvaccinated and vaccinated group. Furthermore it was not enhanced by BCG vaccination providing a theoretical causal link between the reduction of IFNγ observed in the delayed vaccine group and increased T cell production of IL-10. As suppression assays were not performed to assess the function of these cells it is difficult to know if these IL-10 producing T cells are Tr1 Tregs. One of the strategies that mycobacteria have developed to evade the immune system is to downregulate MHC class II molecules which is IL-10 dependent (Sendide, Deghmane et al. 2005) suggesting this may influence the polarisation of the effector memory T cells leading to a reduced Th1 mycobacterial response after BCG vaccination.

The lack of IL-10 production in response to BCG vaccination is in contrast to a

recent detailed multiplex study where IL-10 was strongly (10 – 20 fold) induced by BCG, along with IL-17 in 6 day whole blood assays (Madura Larsen, Benn et al. 2007; Mendez-Samperio, Trejo et al. 2008; Soares, Scriba et al. 2008). Madura Larsen *et al* also showed that BCG matured DCs showed enhanced IL-10 and diminished IL-12 production. These DCs primed naïve T cells to develop into IL-10–producing T cells with no Th1 or Th2 bias. It is interesting to note that IFNγ and IL-10 had completely opposite effects on increasing the dose of BCG with IL-10 increasing with increasing dose (Madura Larsen, Benn et al. 2007). A study in South Africa showed upregulation of IL-10 but to very low levels (< 0.01% of $CD4^+$ or $CD8^+$ T cells) (Soares, AP *et al*, 2008) in 12 hour cultures, whereas our cultures were for 5 days and induced IL-10 in the 1.5 - 4% range for $CD4^+$ and $CD8^+$ T cells in both the BCG vaccinated and unvaccinated groups.

As a group there was no significant induction of IL-10 in response to PPD at 4½ – or 9- months of age following vaccination at birth, however certain individuals responded, in fact 50% elicited increased IL-10 production at 4½ months and 50% had downregulation IL-10 compared to at birth. This heterogeneity in IL-10 responders may be due to the highly polymorphic IL-10 gene. In those being treated for bladder cancer with intravesical BCG, two particular IL-10 polymorphisms have been identified as risk factors for progression (Basturk, Yavascaoglu et al. 2006). IL-10 polymorphisms have also been associated with TB disease and progression (Oral, Budak et al. 2006). In the Gambia, large natural variations in individual responses was found to mycobacterial antigens after BCG vaccination (Finan, Ota et al. 2008) and significant heritability of IFNγ and IL-13 responses to BCG antigens has been illustrated (Newport, Goetghebuer et al. 2004).

In contrast to the cord blood responses, at least part of the IL-10 production observed at 4½ and 9 months was due to $CD4^+$ and $CD8^+$ production, and in most cases there was a larger proportion of $CD8^+$ T cells that produced IL-10 compared to $CD4^+$ T cells. This suggests that adaptive IL-10 producing T cell responses are induced in early life, and these may have an important immunoregulatory role. Irrespective of the source of IL-

10, its effects are usually similar in all infections; IL-10 suppresses macrophage and DC function, thereby limiting Th1 and th2 effector responses. The timing and strength of the response may be determined by the stimulus. Infections of low virulence, such as NTM, may lead to IL-10 induced regulatory T cells allowing persistence within the macrophage. In contrast, a strong stimulus, such as BCG vaccination may induce IL-10 that limits immunopathology during early stages of the response and may not be present later (reviewed in (Couper, Blount et al. 2008)). If the PPD-induced IL-10 observed at all time points, in both groups is due to NTM exposure then maybe this limits the IL-10 producing cells that are induced after BCG vaccination as seen in our results.

Although responses to PPD were observed in cord blood, responses at 4½ months in the unvaccinated group were more robust with increased $CD4^+CD25^+$, $CD4CD25FOXP3^+$ and higher levels of IFNγ and IL-13 after stimulation with PPD most probably primed by exposure to NTM over the preceding 4½ months. By contrast the percentage of proliferating cells and IL-10 remained similar between birth and 4½ months. Ki-67 is a measure of proliferating cells at the time of observation which in our case is at 5 days. We have shown that in unstimulated cord blood cultures 10% of lymphocytes are proliferating which is much greater than proliferation observed at 4½ months (approx 1.5%), however the percentages of $CD4^+$ and $CD8^+$ T cells proliferating was similar between time points at 2% and 4% respectively. We also showed that cord blood cells are more susceptible to cell death. It is possible that cord blood lymphocytes are proliferating at a greater rate because many of them are dying. Therefore comparing cord blood to infant cell responses using phenotyping alone does not fully examine the quality of the responses, and it is important to take into account cell numbers and cell death. There was a trend for IL-10 responses to increase over 4½ months in the unvaccinated group but this was not significant after adjustment for multiple testing although it was still significantly higher than the unstimulated control. The induction of IL-10 observed at birth may be due to *in utero* exposure to mycobacterial antigens or non-specific induction as mentioned earlier.

Our results also suggested that different cells are responsible for producing IL-10 in response to PPD at birth than at later age groups.

It was not expected that the IFNγ response observed at 4½ months in the BCG vaccinated group would wane by 9 months of age. It was not possible to ascertain what effect this has on protection against TB but Black *et al* proposed that the difference in magnitude of PPD response pre- and post- BCG vaccine was likely to be more predictive of BCG efficacy than the absolute value post vaccine. This was observed in Malawi where the reduced difference between pre- and post- BCG reactivity compared to that seen in the UK corresponded to a lack of protection in Malawi and could be due to NTM (Black, Weir et al. 2002). This was also illustrated by additional studies comparing Malawi and the UK. The responses to NTM antigens were similar in both populations after BCG vaccination but were different prior to vaccination (Weir, Black et al. 2006). When comparing the different vaccination schedules in our study, the difference between pre-and post-vaccination was smaller in the delayed vaccine group that would have had exposure to NTMs, compared to the group vaccinated at birth and may indicate reduced protection.

It was interesting to observe a sustained IL-13 production in response to PPD after vaccination at either time points. IL-13 is a Th2 cytokine more often associated with allergic inflammation although also possesses anti-inflammatory properties. It shares a receptor subunit with IL-4 (IL-4Rα) and therefore the function of these cytokines is often linked. Although the role that IL-13 plays in mycobacterial infection is not clear, BCG has been shown to induce IL-13 in cattle in association with anti-mycobacterial activity (Endsley, Hogg et al. 2007) and in human studies IL-13 concentrations are increased in TB patients that are fast responders to treatment (Djoba Siawaya, Beyers et al. 2009). This would suggest that IL-13 has a protective role in mycobacterial infection.

At 4½ months of age, IL-6 was upregulated in response to the mycobacterial antigens with a 138-fold increase in the BCG vaccinated group and a 16-fold increase in

the unvaccinated group. It is often stated that neonates have impaired production of Th1 cytokines (Adkins, B, 2004) but the IL-6 innate cytokine is often enhanced in these young children (Marchini, Berggren et al. 2000; Schultz, Rott et al. 2002). In one report, responses of neonatal (cord blood) monocytes to lipopolysaccharide (LPS;TLR4) were biased towards a high IL-6/TNFα ratio *in vitro*. Compared to adults, neonates produced increased levels of IL-6, but lower TNFα which was contributed to a heat stable neonatal serum factor(s) (Angelone, Wessels et al. 2006). Innate interaction of mycobacteria often involves TLR4 and thus the IL-6 production observed in our study maybe due to an innate response to BCG and NTM through the TLR4 pathway. Further investigation is needed to determine the role of IL-6 in infants.

The strain of both the NTM and the BCG could have a profound effect on the BCG immunogenicity. Aguirre-Blanco *et al* showed that of the three strains (Danish 1331, Glaxo 1077 and Pasteur 1173), it was the Glaxo strain that elicited the most IFNγ production from $CD4^+$ T cells and highest upregulation of $CD25^+$ on CD4 T cells (Aguirre-Blanco, AM *et al*, 2007). The BCG strain used in our study was independently typed and found to be derived from the Russian strain which is the strain closest to the original BCG strain ((Mostowy, Tsolaki et al. 2003; Keller, Bottger et al. 2008)). Several studies have compared the immunogenicity of the different strains and found that, in addition to BCG Glaxo and Pasteur, BCG Russia eliminated recombinant BCG in mice more efficiently than the Japanese and Prague strains (Lagranderie, Balazuc et al. 1996). In a separate study, BCG Russia and BCG Tokyo induced higher levels of NO than other strains in the A549 human lung epithelial cell line but BCG Russia was also more susceptible to the effects of NO-induced growth inhibition suggesting BCG Russia may not survive in the host as long as the other strains (Hayashi, D *et al*, in press). However, the protective efficacy in a guinea pig model of pulmonary tuberculosis with early- (BCG-Japanese) and late- (BCG-Danish, Glaxo, Connaught, Pasteur and Tice) strains of BCG was comparable, with the exceptions of BCG-Glaxo that had a relatively poor efficacy and BCG Tice that

was the most protective vaccine suggesting strain differences are not responsible for the variability of BCG protection (Horwitz, Harth et al. 2009). Persistence of antigen could result in continued activation and may prevent activated cells from developing into memory cells as proposed by Goldsack and Kirman (Goldsack and Kirman 2007) suggesting it may be important to consider strain variation when assessing protection from BCG vaccination.

The inhibitory effect of NTM on the protective efficacy of BCG depends critically on the extent of cross-reactivity of antigens shared with the vaccine. Most studies have shown that BCG vaccination on a background of prior exposure to *M. avium* provided less protection than on a background of more distantly related strains such as *M. vaccae* (Black, Dockrell et al. 2001; Brandt, Feino Cunha et al. 2002; Demangel, Garnier et al. 2005; Weir, Black et al. 2006; Young, Slobbe et al. 2007). A study in The Gambia using mycobacterial skin testing to various environmental mycobacterial antigens showed that *M. intracellulare* is the predominant mycobacterial species (81.2% of BCG naïve 6 – 18 year olds had induration > 3 mm), closely followed by *M avium* (68.3%) present throughout the country, although slight geographical differences within the country were observed (Corrah 1994). This may account for the reduced IFNγ production observed after delaying the BCG vaccine in our study. An interesting study illustrated that BCG vaccination following prior exposure to mycobacteria elicited a quicker memory recall response within the first week of BCG vaccination. Non-sensitised donors exhibited a gradually increasing responsiveness to mycobacterial antigens reaching maximum between day 56 and 365 days post vaccination (Ravn, Boesen et al. 1997). This may be important to our findings that by 9 months there were no differences between the groups but differences may occur up to this point that were not examined.

Responses to EC (antigen used in the commercial IGRA assays) were very low. This is most likely due to a lack of infection in the study participants as was expected in this age group. It was possible that the concentration used in the study (chosen from

previous adult ELISpot assays) was insufficient to induce a detectable immune response in infected infants, however responses above background were observed in all age groups. IFNγ, IL-6 and IL-10 was induced compared to unstimulated cultures in the naïve group that was not apparent in the BCG vaccinated group at 4½ months, however when comparing the two vaccine groups IL-10 was the only cytokine found to be increased in the unvaccinated individuals. It is possible that young children in this setting are being exposed to NTM that contain or are cross primed by EC, such as *M. leprae, M. kansasii, M. marinum, M. smegatus, M. ulcerans*. In a previous study from The Gambia, 30% of adult community controls with no known exposure to *M.tb* responded to ESAT-6 (Vekemans, Lienhardt et al. 2001). In our study the role of the EC specific IL-10 responses in the BCG naïve individuals is unclear. IL-10 has also been shown to correlate with failure of BALB/c mice to control *M. avium* infection, whereas IL-10 ablation enhanced protection in these mice (Roque, Nobrega et al. 2007). This may suggest that reduced IL-10 observed in BCG vaccinated individuals could be linked to protection against *M.tb*.

Overall our studies suggest that BCG vaccination on the background of exposure to NTM is less immunogenic than vaccination at birth eliciting a predominantly Th2 response. We hypothesise that this may in part be due to mycobacterial induced IL-10 production from T cells (possibly by Tr1 induced Tregs). In addition BCG vaccination at birth induced a strong Th1 and Th2 response but only the Th2 response remains by 9 months of age suggesting that although BCG can induce a Th1 response in early life it is transient and the remaining memory response is predominantly Th2 skewed as observed in response to many other vaccines in early life.

CHAPTER 6

Immune correlates of the tuberculin skin test

6.1 INTRODUCTION

6.1.1 The tuberculin skin test (TST)

The TST or Mantoux test is used as a standard diagnostic tool to assess for infection with *M.tb*. The test involves the intradermal injection of PPD-tuberculin (PPD-T) into the volar region of the forearm and the induration (palpable raised hardened area) of the delayed type IV hypersensitivity (DTH) reaction is recorded 48 – 72 hours later. The number of T.U. injected varies from 1 – 10 T.U. according to national guidelines; making it difficult to compare studies across geographical locations (Farhat, Greenaway et al. 2006). In the UK, 2 T.U. (RT23, SSI, Denmark) are often used in standard Mantoux testing (DoH 2006) in immune competent individuals, whereas most of the data from the US are derived from 5 T.U. (PPD-S, Tubersol® or Aplisol®, US) (2000), although 1 T.U. was found to be sufficient to diagnose TB in neonates in the US (Hoskyns, Simpson et al. 1994). Generally a positive skin test is defined as an average induration (mean of width and length) of $\geq$ 5 mm, however there are a number of determinants that affect TST reactivity necessitating different 'cut offs' of induration when assessing for the likelihood of *M.tb* infection.

The TST is highly sensitive but has low specificity. As PPD constitutes a mixture of mycobacterial antigens from *M.tb*, the TST reactivity can be affected by cross reactivity with NTM in the environment and by BCG vaccination. Most studies agree that, in adults, a higher cut off of $\geq$ 10 mm increases the likelihood that the individual has TB infection. The TST does, however not distinguish between LTBI, i.e. infected but asymptomatic, and active TB disease.

6.1.2 Effect of BCG vaccination on TST

BCG vaccination causes a local immune reaction that results in tissue necrosis and long term scarring over the vaccination site. It is not clear if the presence of scarring is associated with immune responses or protection from BCG. By 5 years of age

approximately 54 - 99% of children who received BCG vaccination at birth develop a scar (Baily 1980; Fine, Ponnighaus et al. 1989; Vallishayee, Anantharaman et al. 1998). In Malawi, at 25 - 36 months post BCG vaccination, the average scar size of infants that were vaccinated < 1 month of age was 4 mm, which is lower than those vaccinated at 10-14 years (average 8.5 mm) (Floyd, Ponnighaus et al. 2000). A correlation between a positive TST and BCG scar formation was found in some studies (Sterne, Fine et al. 1996; Lockman, Tappero et al. 1999), but not in others (Sedaghatian and Shana'a 1990; Lienhardt, Fielding et al. 2003) (Garcia-Sancho, Garcia-Garcia et al. 2006) and no association was found between TST and protection against TB (Corrah, Byass et al. 2000), (Sterne, Fine et al. 1996). Interestingly, the presence of a vaccinia (from previous Smallpox vaccination) and/or BCG scar was found to predict lower all-cause mortality, although the mechanism of these non-specific effects is unknown (Aaby, Gustafson et al. 2006).

Tuberculin reactivity after BCG immunisation can vary according to the strain and dose of BCG used (Ashley and Siebenmann 1967; Bunch-Christensen 1977; Karalliedde, Katugaha et al. 1987; Wang, Turner et al. 2002; Davids, Hanekom et al. 2006), the method of vaccine administration (Davids, Hanekom et al. 2006), the time since vaccination (Lifschitz 1965; Landi, Ashley et al. 1967; Guld, Waaler et al. 1968; Horwitz and Bunch-Christensen 1972; Karalliedde, Katugaha et al. 1987; Ormerod and Garnett 1988; Menzies and Vissandjee 1992; Teale, Cundall et al. 1992; Menzies 2000), the number of BCG vaccinations administered (Ildirim, Hacimustafaoglu et al. 1995) and the age, weight and nutritional status of the child at the time of vaccination (Marcus and Khassis 1965; Joncas, Robitaille et al. 1975; Sinha and Bang 1976; Karalliedde, Katugaha et al. 1987; Menzies and Vissandjee 1992; Ormerod and Garnett 1992; Menzies 2000; Okan, Karagoz et al. 2006; Richeldi 2006) and genetic variation between populations (Sepulveda, Heiba et al. 1994; Sepulveda, Heiba et al. 1994; Newport, Goetghebuer et al. 2004)).

Although there are many conflicting studies on the effect of BCG on long term

reactivity of the TST, BCG vaccination at birth appears to result in TST reactivity ≥ 10 mm for 6 – 12 months (Lockman, Tappero et al. 1999; Lienhardt, Sillah et al. 2003; Santiago, Lawson et al. 2003; Ota, Goetghebuer et al. 2006; Chan, Chang et al. 2008). However other studies have suggested that the peak of TST reactivity (≥ 10 mm) occurs at about 2 months of age and then gradually decreases to 12 months of age after which a TST ≥ 10 mm is more specific for TB infection. However, a TST result between 5 and 10 mm persists longer after BCG vaccination (Miret-Cuadras, Pina-Gutierrez et al. 1996; Bozaykut, Ipek et al. 2002; Reid, Ward et al. 2007). Miret-Cuadras *et al* showed that even 20 - 25 years after vaccination at birth a higher proportion of individuals (53%) had a TST ≥ 5mm compared to non-vaccinated individuals (17.4%), although TST ≥ 15 mm was more likely to represent TB disease (Miret-Cuadras, Pina-Gutierrez et al. 1996). A longitudinal study in Taiwan defined age specific 'cut offs' to identify LTBI in children up to 7 years of age. The effect of neonatal BCG on TST declined from 3 months to 7 years therefore optimal TST 'cut offs' for ages 0 – 1, 2 – 3, 4 – 5 and 6 – 7 years were defined as 21, 18, 13, and 10 mm respectively (Chan, Chang et al. 2008). Interestingly, BCG vaccination given after infancy (> 1 year of age) was twice as likely to result in a TST ≥ 5 mm 20 years later, than when given to newborns (Menzies and Vissandjee 1992; Wing, Ekmark et al. 2002; Farhat, Greenaway et al. 2006).

6.1.3 Effect of NTM on TST

Exposure to NTM in different geographical locations has been shown to affect the TST although rarely causes an induration of >10 mm (Edwards, Acquaviva et al. 1969; Farhat, Greenaway et al. 2006). A meta-analysis that assessed the contribution of BCG and NTM on TST reactivity reported that in Montreal only 0.1% of TST responses >10 mm could be attributable to exposure to NTM, *M. intracellulare* (prevalence 2-8%) compared to 2.3% in India where the prevalence of the same NTM was 86% (Farhat, Greenaway et al. 2006). NTM exposure has been assessed in studies in Malawi, by measuring IFNγ

responses to mycobacterial antigens *in vitro*. Individuals with no evidence of a BCG scar exhibited responses particularly to mycobacteria from the MAIS complex (*M. avium, M. intracellular, M. scrofulaceum*) (Black, Fine et al. 2001; Black, Weir et al. 2003). A study in The Gambia showed exposure to NTM in BCG naïve children (6 – 18 years if age) throughout the country with a predominance of *M. intracellulare* (81.2%) and *M. avium* (68.3%) and less prevalence of *M. marinum* (28.4%) (Corrah 1994). Infants can also respond to NTM, 22% (6/27) of BCG naïve 4½ month old infants in The Gambia had a TST > 5 mm suggesting NTM priming (Ota, Goetghebuer et al. 2006).

As discussed in the main introduction (Chapter 1) many studies have assessed the contribution of NTM exposure to BCG efficacy and TB protection. Most animal and human studies agree that there are interactions between NTM and BCG due to the sharing of mycobacterial antigens, but there are conflicting views on whether exposure enhances (Pabst, Godel et al. 1989), inhibits (Palmer and Long 1966; Edwards, Goodrich et al. 1982; Orme and Collins 1984; Black, Dockrell et al. 2001; Brandt, Feino Cunha et al. 2002; Buddle, Skinner et al. 2002; Howard, Kwong et al. 2002; de Lisle, Wards et al. 2005; Young, Slobbe et al. 2007) or has no effect (Ravn, Boesen et al. 1997) on the function of BCG. This may be due to widespread differences in the load and strain of NTM in different populations, but many other reasons may account for these observations.

6.1.4 Alternative diagnostic test for TB

Because of the potential for inaccuracy of the TST, alternative diagnostic tests have been developed based on advances in mycobacterial genomics. Two antigens, early secreted antigenic target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10), have been found to be present in *M.tb* but not in BCG (Mahairas, Sabo et al. 1996; Lalvani, Pathan et al. 2001). Two commercial tests are now available based on enzyme-linked immunospot assay, ELISpot (T-SPOT.*TB*; Oxford Immunotec, Oxford, UK) and enzyme-linked immunosorbent assay, ELISA (QuantiFERON-TB Gold; Cellestis, Carnegie, Australia)

based on ESAT-6 or CFP-10 induced IFNγ production from T cells. Both these interferon-gamma release assays (IGRA) have been shown to be more specific than TST in diagnosing TB disease and in LTBI subjects (detected using case contact studies) and more sensitive than TST in TB cases (Lalvani 2007; Chun, Kim et al. 2008; Connell, Ritz et al. 2008; Mandalakas, Hesseling et al. 2008; Stephan, Wolf et al. 2008). These studies have also shown that the IFGA tests are not affected by BCG vaccination or NTM exposure (Dheda, Udwadia et al. 2005). Nienhaus *et al* showed that 85% of $TST^{+}/IGRA^{-}$ discordant results were due to BCG vaccination (Nienhaus, Schablon et al. 2008). As mentioned earlier in the introduction of this chapter, ESAT-6 and CFP-10 are not present in BCG but they are present in some species of NTM including *M. leprae, M. kansasii, M. marinum, M. smegmatis, M. ulcerans* (Demangel, Garnier et al. 2005) that could influence the IFGA tests in areas where these NTM are prevalent.

Results have varied when analysing correlations between IFNγ production in response to PPD and the TST although greater concordance was found comparing responses to the ESAT-6/CFP-10 antigen with the skin test (Converse, Jones et al. 1997; Desem and Jones 1998; Streeton, Desem et al. 1998; Elliott, Hurst et al. 1999; Pottumarthy, Morris et al. 1999; Black, Fine et al. 2001; Mazurek, LoBue et al. 2001; Ota, Goetghebuer et al. 2006; Martins, Lima et al. 2007). It is however recognised that IGRA assays using specific antigens are less prone to the confounder effect of BCG vaccination and NTM than the TST.

Immune correlates of protection against TB are unclear, but recent evidence suggests that IFNγ is not the only cytokine that is involved (discussed in Chapter 1). In addition, immune responses involved in the TST are not well studied and therefore this chapter aims to examine the immune correlates of TST reactivity at 4½ months of age and to further understand the contribution BCG and NTM have in this reactivity by comparing infants who have been BCG vaccinated at birth (Group 1) to BCG naïve children (Group

2). It further analyses the relationship between pro-inflammatory and anti-inflammatory cytokines in order to give additional insight into how the TST is controlled and regulated.

6.2 RESULTS

6.2.1 Reactivity to the TST at 4½ months was related to BCG vaccination

All infants were given a 2 T.U. tuberculin skin test at 4½ months of age. This included the BCG vaccinated group (Group 1, n = 51) and the BCG naïve infants (Group 2, n = 39). The latter group provided an opportunity to assess for the effect of exposure to NTM to TST reactivity. Induration of ≥ 10 mm in either group was treated as suspected LTBI or TB disease and investigated further (see Chapter 2: Materials and Methods).

Surprisingly, all the subjects in the unvaccinated group (Group 2) exhibited a completely anergic (zero induration) response to the TST (Figure 6.1A). Within the BCG vaccinated group 47% (24/51) of subjects exhibited a positive response (≥ 5 mm) and the remaining 53% were predominantly anergic. Of those that had ≥ 5 mm induration, 58% (14/24) had responses ≥ 10 mm and were investigated further for possible TB exposure. It was found that two of these children had potentially been exposed to TB in their compound and they will be discussed later in this chapter. No child had a TST response greater than 15 mm (Figure 6.1A). Skin test induration in Group 1 did not follow a normal distribution. Apart from the prominent peak at zero of induration there were three further peaks at 7, 10 and 13 mm of induration (Figure 6.1B).

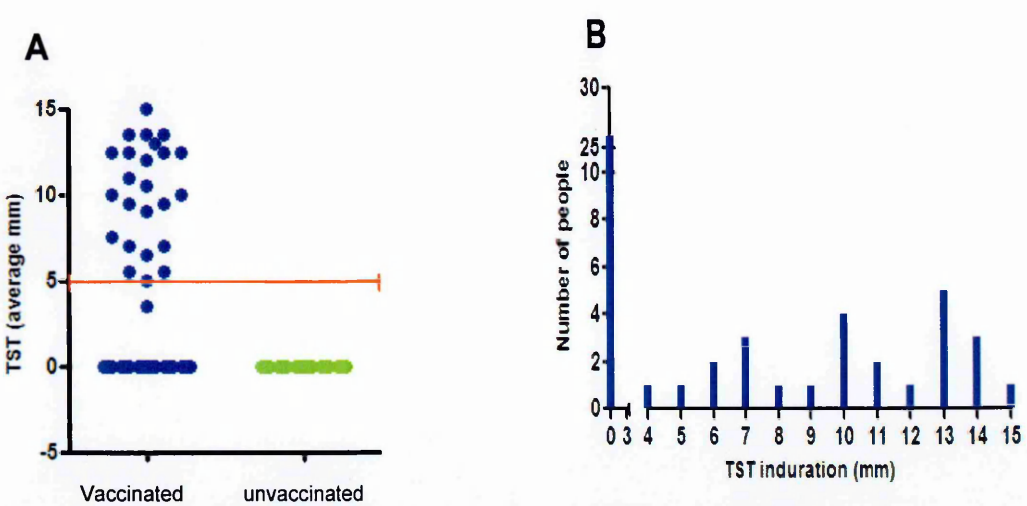


Figure 6.1: Tuberculin Skin Test (TST) results at 4½ months of age. *2 T.U PPD was*

injected into the forearm of each individual at 4½ months of age. The average length x width (mm) of the induration was measured 48 – 72 hours later. (A) Average TST induration for each group, red line represents a 5 mm 'cut off' for positive reactivity, (B) frequency graph of TST induration. Vaccinated (Group 1) n = 51, unvaccinated (Group 2) n = 39.

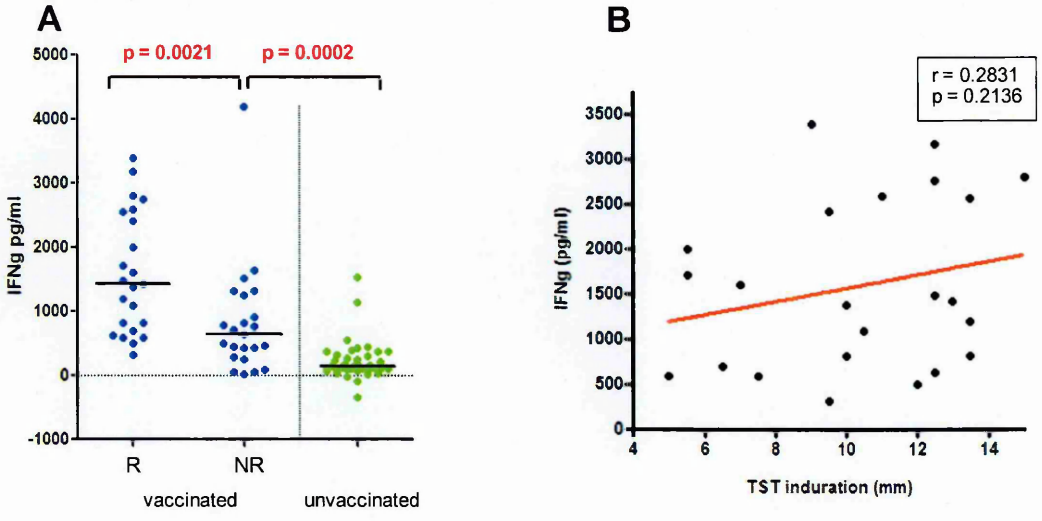
6.2.2 PPD and BCG specific cytokine responses and TST reactivity

The Mantoux reaction is caused by a local cell mediated immune response to PPD at the site of injection, but it remains unclear as to how Mantoux reactivity relates to mycobacterial activity in peripheral blood. Within the BCG vaccinated group, the responders (≥ 5 mm, denoted R) had higher day 5 whole blood IFNγ reactivity to PPD than the non-responders (< 5 mm, denoted NR) (Figure 6.2A, p = 0.0021). Even the NR in the vaccinated group, most of whom were anergic, still exhibited greater IFNγ reactivity than the anergic BCG naïve individuals from Group 2 (p = 0.0002) suggesting that IFNγ production is upregulated in response to BCG vaccination even if it does not lead to a positive TST. IFNγ was also increased in the responders after *in vitro* stimulation with the BCG vaccine compared to the non-responders (p = 0.0349). There was a trend for greater IFNγ production from the non-responders compared to Group 2 but this was not significant (p = 0.1575). None of the other cytokines tested (IL-13, IL-6, IL-7 and IL-17) were significantly different between responders and non-responders in response to PPD or BCG. The borderline significantly higher IL-6 to these antigens in responders (PPD; p = 0.0560, BCG; p = 0.0221) was lost after Bonferroni correction for multiple testing (data not shown). Although TST responders produced more IFNγ compared to non-responders, the quantity of IFNγ produced and the size of induration of the TST reaction was not correlated (Spearmans r = 0.2831, p = 0.2136) (Figure 6.2B).

The anti-inflammatory cytokine, IL-10 was upregulated in response to PPD in both groups (vaccinated and unvaccinated) at 4½ months compared to unstimulated cultures, but there was no difference between the two groups ($p = 0.965$) suggesting that BCG vaccination does not induce PPD specific IL-10. This mycobacterial specific IL-10 in the naïve group is most likely to be the result of exposure to NTM.

Interestingly, the quantity of IL-10 produced in response to PPD at 4½ months of age inversely correlated to the induration of the TST ($r = 0.4396$, $p = 0.0358$) (Figure 6.2D) which would suggest that exposure to NTM-induced IL-10 can influence the skin test reactivity. If this was true one might predict that those with an anergic TST would have the highest levels of IL-10. However, IL-10 production was comparable between responders and non-responders and the unvaccinated groups (Figure 6.2C and D). However the distribution of data was quite different between groups (Figure 6.2C). Only 3 BCG vaccinated responders (13.0%) produced greater than 34 pg/ml (3 x SEM) of IL-10 in response to PPD whereas 18.2% (4/22) of the non-responders and 26.3% (10/38) of the unvaccinated individuals responded with higher levels of IL-10 production to PPD. Furthermore, those individuals that downregulated IL-10 production (PPD stimulated minus unstimulated control was < zero) in response to PPD stimulation were predominantly TST responders; 83% (5/6) compared to none of the non-responders and one of the unvaccinated. A similar trend was also observed when comparing *in vitro* reactivity to the BCG imunogen.

IFNγ



IL-10

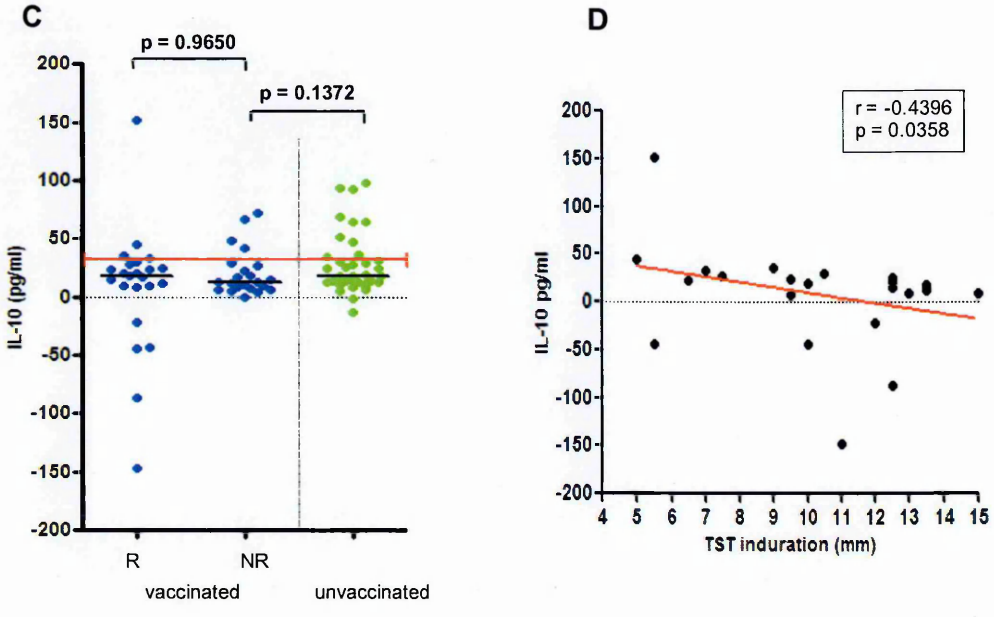


Figure 6.2: Relationship between TST and cytokine production. *At 4½ months of age, whole blood was incubated with PPD for 5 days. IFNγ (A and B) and IL-10 (C and D) cytokine production was measured in the supernatants of the cultures. Unstimulated control was subtracted from the PPD stimulated values, black bar represents the median value, red line represents 34 pg/ml (3 x SEM). A Mann Whitney U test at 5% significance was applied to compare the cytokine production between groups. Correlation between the*

TST induration of the responders and IFNγ (B) and IL-10 (D) was calculated using Spearmans correlation coefficient at 5% significance. R = responder n = 23, NR = non responder n = 22, unvaccinated n = 38.

Neither IFNγ nor IL-10 produced in day 5 BCG culture supernatants correlated with TST induration. However there was a trend for the highest cytokine production of IFNγ to fall between 9 – 12 mm induration. In contrast, the lowest IL-10 production was observed at this same induration (Figure 6.3A and B). One individual (BCG033) exhibited the highest IFNγ response alongside the lowest IL-10 production, indicated with an 'X' in Figure 6.3A and B. Interestingly this individual corresponded to the high IFNγ/ low IL-10 responder to PPD. This may indicate the different influences that may confound the TST reactivity and would be worth examining in more detail in a later study. There were also no significant correlations between the other cytokines tested (IL-6, IL-13, IL-7 and IL-17) from PPD or BCG cultures and the TST induration.

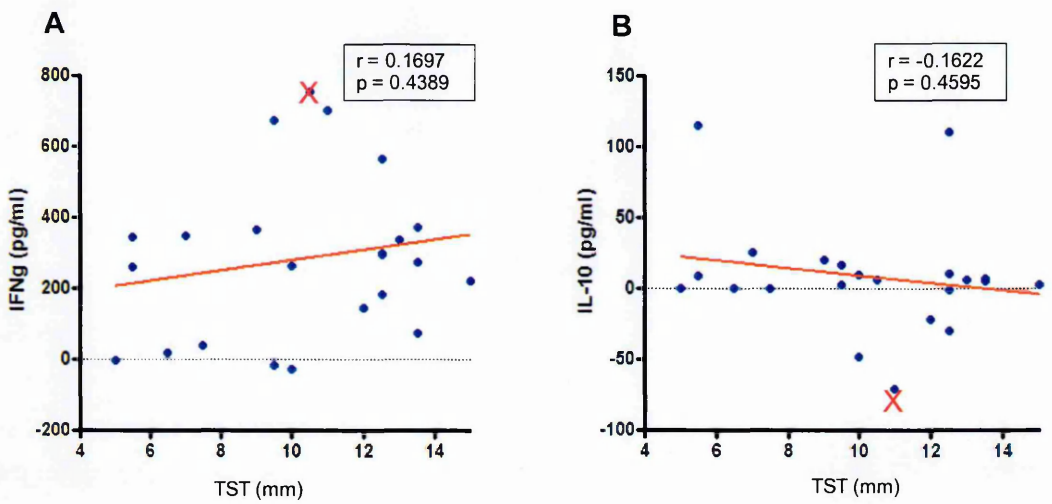


Figure 6.3: In vitro responses to BCG vaccine compared to TST induration in Group 1 (vaccinated). *At 4½ months of age, whole blood was incubated with BCG for 5 days. IFNγ and IL-10 cytokine production was measured in the supernatants of the cultures. Unstimulated control was subtracted from the PPD stimulated values, black bar represents*

the median value. Correlation between the TST induration of the responders and IFNγ (A) and IL-10 (B) was calculated using Spearmans correlation coefficient at 5% significance. n = 23. X = the corresponding results for one subject.

Studies have shown a reduced ratio of IFNγ: IL-10 in response to PPD in TB patients compared to *M.tb* exposed but healthy individuals (Hussain, Talat et al. 2007). We examined this ratio in the context of BCG vaccination and found BCG vaccinated infants had a higher IFNγ: IL-10 ratio compared to unvaccinated infants but there were no differences in IFNγ: IL-10 ratios according to TST reactivity (Figure 6.4A and B). This is likely to reflect the increased IFNγ but lack of IL-10 induction observed in BCG vaccinated individuals.

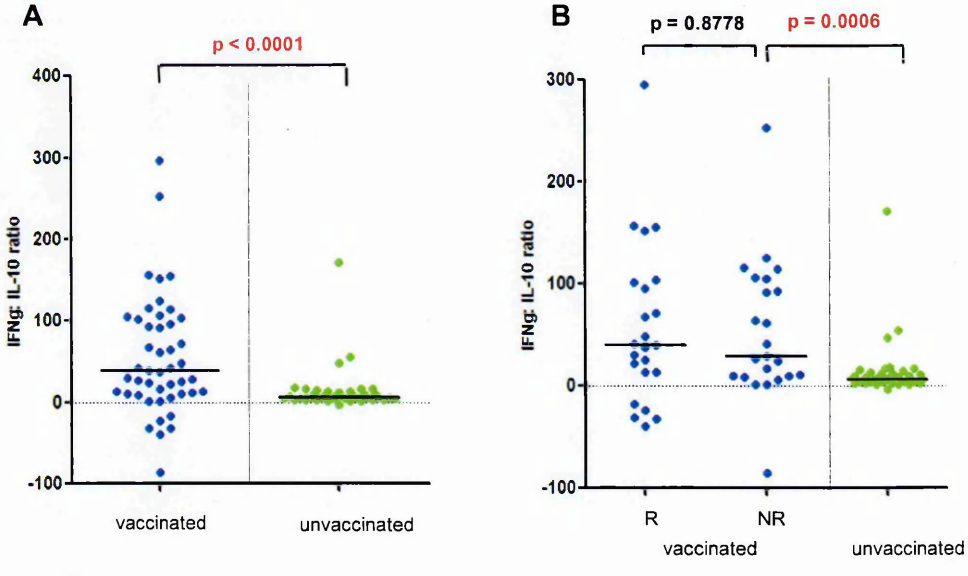


Figure 6.4: IFNγ to IL-10 ratio in response to PPD stimulation. *At 4½ months of age, whole blood was incubated with PPD for 5 days. Unstimulated control was subtracted from the PPD stimulated values, black bar represents the median value. The ratio of IFNγ and IL-10 cytokine production was measured in the supernatants. A Mann Whitney U test at 5% significance was applied to compare the ratio of IFNγ: IL-10 between groups (A) and between TST responders and non-responders and between non-responders and*

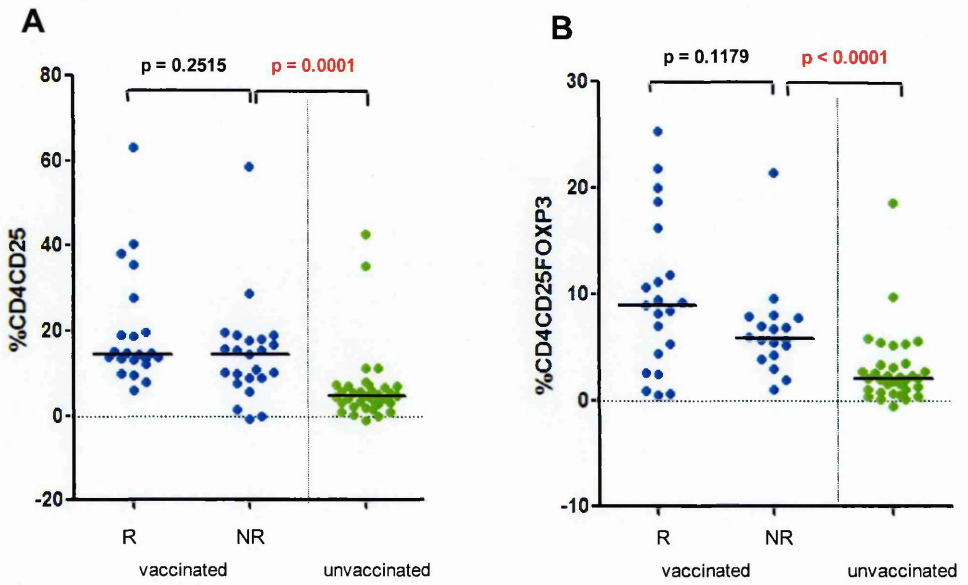
unvaccinated (B). R = responder n = 23, NR = non responder n = 22, unvaccinated n = 38.

6.2.3 PPD and BCG specific T cell responses by flow cytometry and TST reactivity

It was hypothesised that the level of Tregs circulating in the peripheral blood may influence the reactivity to the skin test, however a comparison of the *ex vivo* FOXP3 Tregs ($CD4^+CD25^+FOXP3^+$) between responders and non responders in the BCG vaccinated group showed similar levels ($p = 0.7341$) which were also similar to those of the unvaccinated group.

Although PPD stimulated IFNγ production was greater in the TST responders, this did not correspond to greater T cell activation ($CD4^+CD25^+$) or proliferation ($Ki\text{-}67^+$) compared to the non-responders but did remain significantly higher than the unvaccinated group (Figure 6.5A and B). However, with respect to *in vitro* stimulation with BCG there was a trend for activated T cells ($CD4^+CD25^+$) and Tregs ($CD4^+CD25^+FOXP3^+$) to be upregulated in those that responded to the TST ($p = 0.0785$ and $p = 0.0696$ respectively) (Figure 6.5C and D).

PPD



BCG

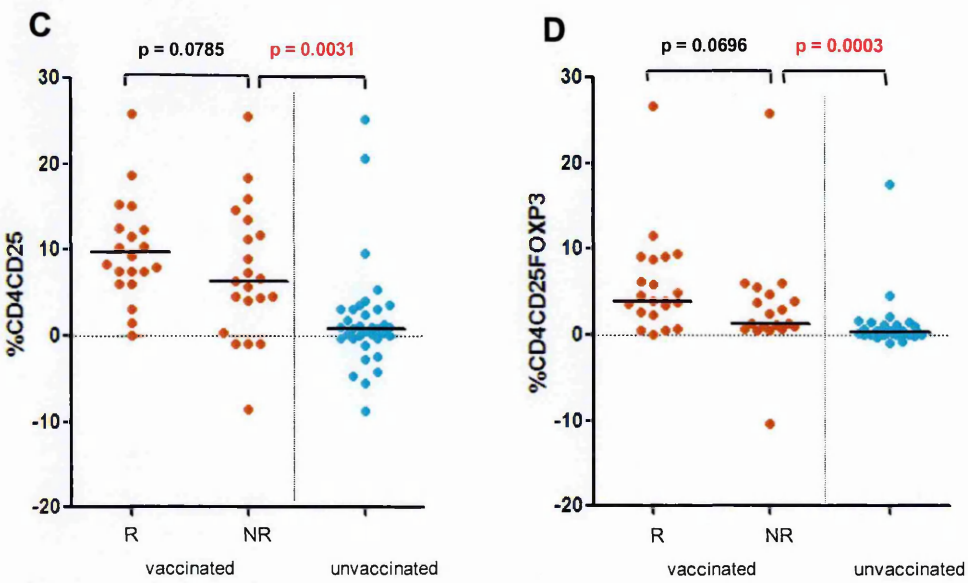
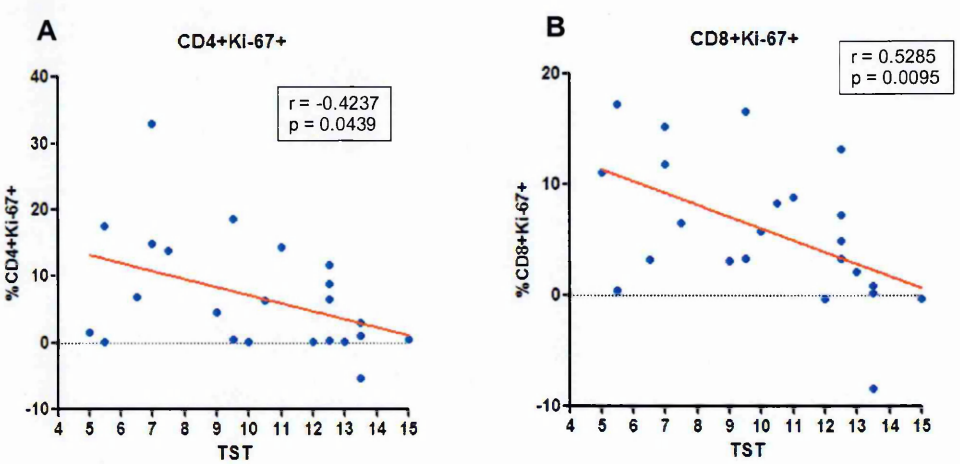


Figure 6.5: Relationship between TST and T cell phenotype in response to PPD and BCG. *At 4½ months of age, whole blood was incubated with PPD (A, B) and BCG (C, D) for 5 days. Unstimulated control was subtracted from the PPD stimulated values, black bar represents the median value. Activated T cells ($CD4^+CD25^+$) (A, C) and Tregs ($CD4^+CD25^+FOXP3^+$) (B, D) were measured with respect to TST reactivity. Comparisons*

between responders (R), non responders (NR) and unvaccinated individuals were calculated using a Mann Whitney U test at 5% significance. R = responder n = 22, NR = non responder n = 21, unvaccinated n = 34.

Interestingly there was an inverse correlation between the size of the TST and proliferation of both $CD4^+$ and $CD8^+$ T cells after *in vitro* stimulation with BCG, but not after PPD stimulation (Figure 6.6A and B). As observed with IL-10 cytokine production, this correlation was not reflected by significant differences when comparing R and NR. The proportion of proliferating CD8 T cells were, in fact, greater in the R compared to the NR (Figure 6.6C and D). When adjusting for multiple comparisons these observations are no longer significant but it is worth considering these trends in subsequent studies.

BCG



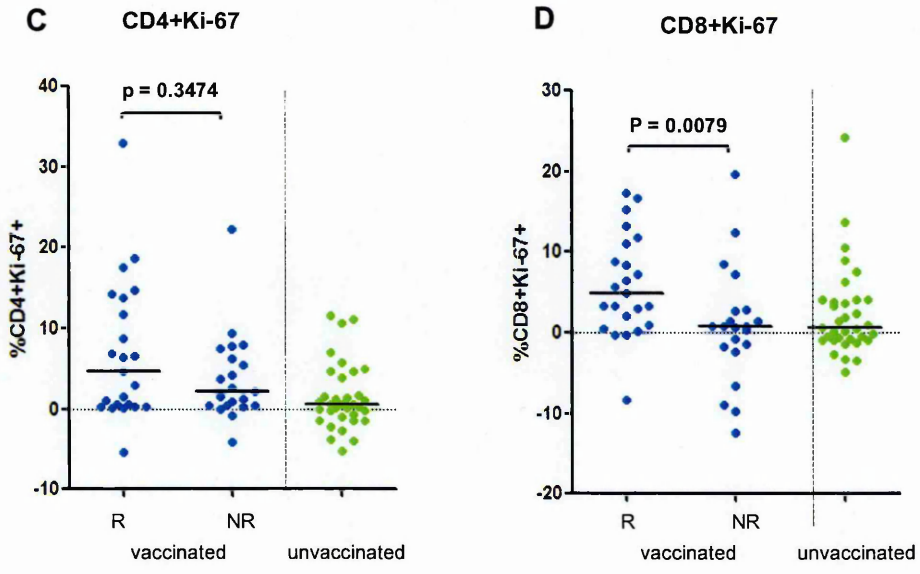


Figure 6.6: In vitro proliferative responses to BCG correlated with TST. *At 4½ months of age, whole blood was incubated with PPD for 5 days. Unstimulated control was subtracted from the PPD stimulated values, black bar represents the median value. Frequency of proliferating $CD4^+$ T cells (A, C) and $CD8^+$ T cells (B, D) was measured using intracellular staining with Ki-67 antibody. Correlation between the TST induration of the responders and $CD4^+Ki\text{-}67^+$ (A) and $CD8^+Ki\text{-}67^+$ (B) was calculated using Spearmans correlation coefficient at 5% significance. Comparisons between responders (R) and non responders (NR) were calculated using a Mann Whitney U test at 5% significance (C, D). R = responder n = 23, NR = non responder n = 21, unvaccinated n = 34.*

T cell production of IL-10, as defined by intracellular staining (ICS) did not differ between responders and non-responders or compared to unvaccinated individuals both in response to PPD and BCG. Furthermore, ICS IL-10 production was not correlated to TST induration as was observed with IL-10 production in the supernatant of cultures, suggesting a non-T cell source for the latter IL-10 levels (Figure 6.7A – D). Antigen

specific TGFβ production by intracellular staining of T cells were very low, in most cases < 1% of T cells produced this cytokine. Comparisons between responders and non-responders did not reveal any significant differences and TGFβ production did not correlate to TST induration (data not shown).

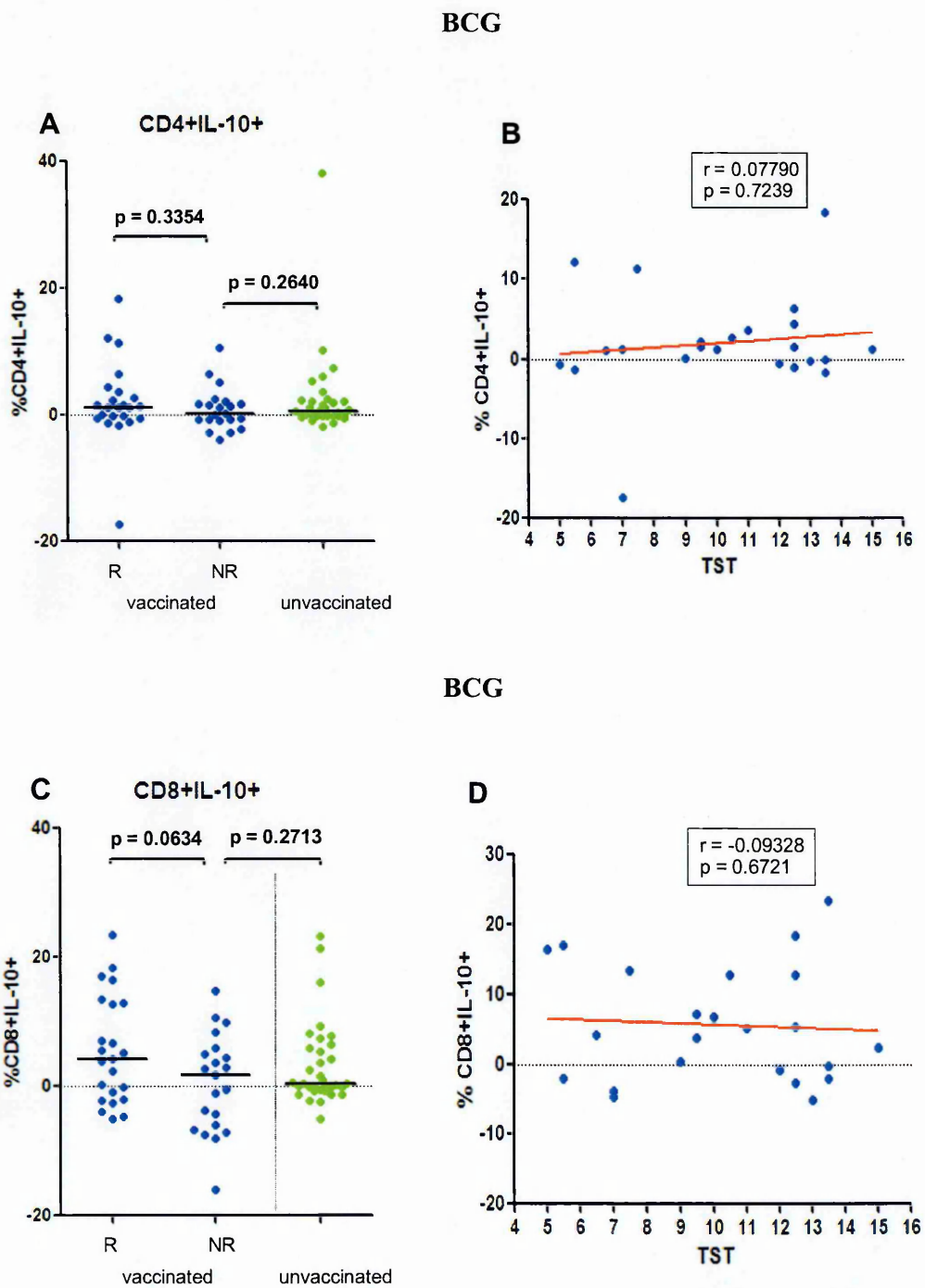


Figure 6.7: Day 5 intracellular IL-10$^+$ T cells in response to BCG correlated with TST. *At 4½ months of age, whole blood was incubated with BCG for 5 days. Frequency of IL-10*

producing $CD4^+$ T cells (A, B) and CD8 T cells (C, D) was measured by intracellular staining. Unstimulated control was subtracted from the BCG stimulated values, black bar represents the median value. Comparisons between responders (R) and non responders (NR) were calculated using a Mann Whitney U test at 5% significance (A, C). Correlation between the TST induration of the responders and $CD4^+IL\text{-}10^+$ (B) and $CD8^+IL\text{-}10^+$ (D) was calculated using Spearmans correlation coefficient at 5% significance. R = responder n = 23, NR = non responder n = 21, unvaccinated n = 34.

6.2.4 ESAT-6/CFP-10 specific responses and TST reactivity

Many studies have indicated that, in the future, IGRA involving the EC antigen complex may replace the TST in determining *M.tb* infection and TB disease. A comparison between *in vitro* EC responses and the TST would help clarify these conflicting studies.

There were no TST reactors in the BCG naïve group therefore it is likely that a TST ≥ 10 mm (observed in 27.5% of Group 1 individuals) was due to the BCG vaccination rather than *M.tb* infection.

Immune responses to EC were compared between responders and non-responders using the same definitions as before. There were no significant differences between the two groups for all the parameters examined including $CD4^+CD25^+$, $FOXP3^+$Tregs, ICS Ki-67, IL-10, TGFβ and production of cytokines (IFNγ, IL-6, IL-13, IL-10, IL-7, IL-17) in the supernatant. However there was a significant increase in IL-10 production from the EC stimulated supernatants in the BCG naïve group compared to the non-responders in the BCG vaccinated group (Figure 6.8A) supporting the previous results from PPD stimulated cultures. EC-specific IFNγ production showed a trend for an inverse correlation with respect to TST induration as illustrated in Figure 6.8B but with a peak of IFNγ production between 9.5 and 12.5 mm, which was seen in immune responses to BCG earlier.

ESAT-6/CFP-10 fusion protein

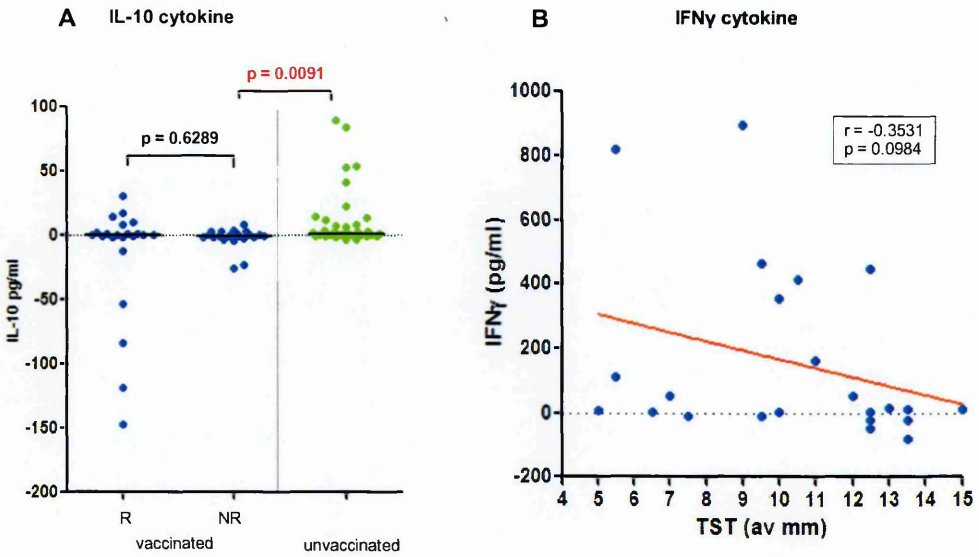


Figure 6.8: Cytokine production in response to ESAT-6/CFP-10 fusion protein compared to TST reactivity. *At 4½ months, whole cord blood was incubated with ESAT-6/ CFP-10 (EC) for 5 days. Cytokine production was measured in the supernatants of the cultures. Unstimulated control was subtracted from the EC stimulated values, black bar represents the median value. A Mann Whitney U test at 5% significance was applied to compare the cytokine production between responders (R) and non- responders (NR) to the TST (A). R= responder n = 23, NR = non responder n = 23, unvaccinated n = 39. Correlation between the TST induration of the TST responders and IFNγ production in response to EC was calculated using Spearmans correlation coefficient at 5% significance. n = 23 (B).*

6.2.5 Suspected TB exposure

TB exposure was assessed by the monthly TB questionnaire (TBQ) and by the TST at 4½ months. From the skin test results 14 subjects had a TST result ≥ 10 mm which could suggest exposure to TB. In all cases all members of the compound were skin tested and chest X-ray performed on all those with a TST ≥ 10 mm. Only two children were identified from this process as probable TB exposure that might account for the high

reactivity to the TST (Table 6.1A).

The TBQ also identified a further two children with potential exposure to TB; one was in the delayed BCG vaccine group and identified as at risk at 1 month of age. This child was therefore dropped out of the study, assessed by the paediatrician and BCG vaccinated immediately. The second child was detected after BCG vaccination and therefore remained in the study (Table 6.1A).

A

Subject ID	Group	Exposure identified by	Age identified (m)	TST at 4½ m	TST at 24 m	Details	Dropped out
BCG005	1	TST	4½	10	4	Member of compound being treated for TB	no
BCG016	1	TST	4½	13.5	0	Suspected case of child in compound but was not confirmed	no
BCG025	2	TBQ	1	N/A	N/A	Father treated during mother's pregnancy	yes
BCG057	2	TBQ	7	0	0	Father currently being treated for TB	no
BCG043	2	TST	20-28	0	19.5	Currently being tested for TB	no
BCG050	1	TBQ	20-28	0	0	Sister had suspected TB	no

B

	Number of individuals	Number of individuals with scar
Anergic (0)	71 (83.5%)	66/72 (91.7%)
1 - 4 mm	2 (2.4%)	1/2 (50%)
5 – 9 mm	7 (8.2%)	6/7 (85.7%)
≥ 10 mm	4 (4.7%)	4/4 (100%)

Table 6.1: TB exposure identified during the study. *(A) Details of individuals identified as having potential exposure to a TB case by TST at 4½- and 20 months of age, (B) At 20 months of age TST results and scar formation in individuals with specific TST indurations (n = 85).*

6.2.6 Repeated TST at 20 months of age

A repeat Mantoux test of all subjects at 20 - 28 months of age was undertaken. By this age the TST response to BCG vaccination would be expected to have waned, and reactivity ≥ 10 mm would be more likely to be due to *M.tb* infection.

Seventy two of the 85 children (84.7%) were anergic to the TST (induration of zero). Of the 13 individuals that exhibited a response, 4 individuals had an induration of ≥ 10 mm (Table 6.1B). These individuals were followed up for suspected TB exposure and one of them was identified as living in a compound of a possible TB index case (Table 6.1A). One individual was also identified through the TBQ that was also completed at 20 months of age. Only one of the three subjects that were previously identified as being exposed to TB at 4½ months had reactivity to the TST and this was < 5 mm (Table 6.1A).

When comparing the TST results to those at 4½ months, it was obvious the timing of the BCG vaccination did not affect the TST at 20 months of age, with similar numbers of positive subjects in both groups (Group 1: 13.3%; Group 2: 12.5%) (Figure 6.9A and B). Scar formation was recorded at this 20 month visit and was present in 91.8% of individuals with a median diameter of 3 mm. Seven out of eighty five (8.2%) children did not develop a scar to BCG vaccination. There was no relationship between lack of BCG scar and timing of BCG vaccination.

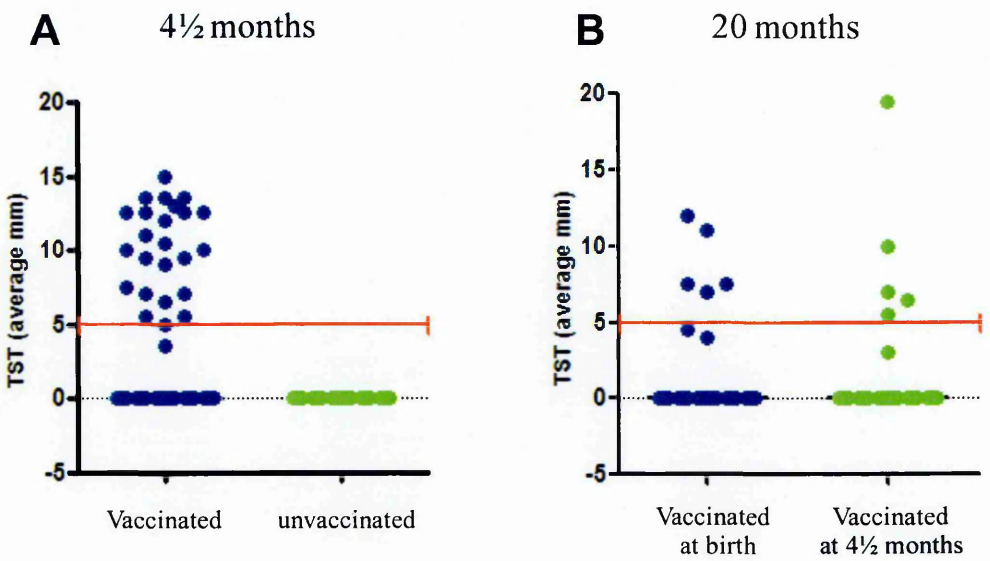


Figure 6.9: TST results at 4½ and 20- months of age. *(A) Average TST induration for each group at 4½ months of age (vaccinated group n = 51, unvaccinated group n = 39), and (B) 20 months of age (Group 1 vaccinated at birth n = 45, Group 2 vaccinated at 4½ months n = 40), red line represents a 5 mm 'cut off' for positive reactivity.*

6.3 DISCUSSION

In concordance with the literature, BCG vaccination induced reactivity to the tuberculin skin test 4½ months later. Studies by Chan *et al* suggest that the 'cut off' for *M.tb* specific reactivity up to 1 year of age was 21 mm and anything below this could be attributed to BCG vaccination. By 20 months of age, the proposed 'cut off' was 18 mm (Chan, Chang et al. 2008). At 4½ months of age none of our subjects had reactivity above 15 mm suggesting that none of the study participants were infected with *M.tb*. At 20 months, 4/85 (4.7%) individuals had a TST $\geq$ 10 mm but only one subject reached a reactivity of $\geq$ 18 mm. This child was indeed identified as being exposed to *M.tb*. However there were also children at 4½ months and 20 months that had an anergic skin test, but were deemed to have had a significant TB exposure by TBQ. This would suggest that these individuals had not become latently infected even though they had been exposed. All children were from the same environment (within a 40 km radius of the Sukuta district) and were recruited randomly throughout the different seasons of the year for two years (June 2006 – May 2008).

In a previous Gambian study Ota *et al* found 22% of BCG naïve children at 4½ months of age had a positive TST response (Ota, Goetghebuer et al. 2006). In the present study none of the unvaccinated individuals reacted to the TST despite having *in vitro* reactivity to PPD and BCG albeit at significantly lower levels than vaccinated children suggesting the skin test may be less sensitive than the 5 day whole blood assays. It may also suggest that there is a defect in the ability of T cells to home to the skin. The Th2 cytokine IL-4 has been shown to downregulate the homing molecule cutaneous lymphocyte-associated antigen (CLA) on virus-specific $CD8^+$ T cells. In contrast IL-12 and, surprisingly IL-10 both increased the expression of CLA (Seneviratne, Jones et al. 2005). In addition differential effects of IL-12 and IL-10 have also been found on superantigen-induced expression of CLA where addition of IL-12 augmented the expression of CLA but IL-10 strongly decreased it (Sigmundsdottir, Johnston et al. 2004).

It is possible that the cytokine milieu induced by exposure to NTM downregulates the homing molecules on PPD-specific T cells thus reducing the TST reactivity.

It was interesting that more than half (53%) of the BCG vaccinated children had an anergic skin test at 4½ months of age. The likelihood of false negative results was considered. To eliminate the possibility of inactive PPD reagent, the same batch of PPD was used and was tested at the start and half way through the study on a known positive responder. Training of field staff and using two independent readers reduced false readings due to lack of correct skills. False negative TST results can be caused by a number of factors including immunosuppression (e.g. by HIV infection) (Pesanti 1994; Raby, Moyo et al. 2008), age (reduced TST reactivity has been observed in the very young and the very old) (Rodysill, Hansen et al. 1989; Shingadia and Novelli 2003), overwhelming TB infection (Boussiotis, Tsai et al. 2000; Magnani, Confetti et al. 2000), other viral (Starr and Berkovich 1964; Pesanti 1994), or bacterial infections (Mitchell 1935) and recent viral vaccinations (Brody and McAlister 1964; Brody, Overfield et al. 1964; Starr and Berkovich 1964). In TB patients Vdelta2$^+$ T cells are thought to play a role in this anergy (Szereday, Baliko et al. 2008). As mentioned in Chapter 2: Materials and Methods, the prevalence of HIV in The Gambia is 2.4% in adults (aged 15 – 49) and an estimated 1,200 children (aged 0 – 14 years, 0.1% of population) living with HIV (UNICEF 2005) suggesting that it is unlikely that any of the children in this study would be HIV$^+$ and therefore would account for a false negative TST. Twin studies have found conflicting results as to the inheritability of the TST response and therefore it remains unclear if there is a genetic component to the skin test response (Gonzalez, Heiba et al. 1994; Sepulveda, Heiba et al. 1994; Sepulveda, Heiba et al. 1994).

A trend of higher levels of IL-10 production was observed in TST non-responders and BCG naïve anergic individuals compared to TST responders (although overall responses were similar) and within the responders there was an inverse correlation between

higher IL-10 and lower TST reactivity suggesting that IL-10 plays a role in reactivity to TST in relation to BCG vaccination. This was most evident in BCG and EC *in vitro* responses which suggest specificity of the response. The differences observed were mainly from IL-10 production measured in the supernatant of the 5 day cultures and did not correspond to the IL-10 levels measured in the lymphocyte population of cells by flow cytometry, suggesting the IL-10 that influences the TST was produced by non-lymphoid sources, such as macrophages. TST anergy occurs in about 25% of immunosuppressed TB patients which often correspond to poor prognosis (Boussiotis, Tsai et al. 2000; Delgado, Tsai et al. 2002). This anergy has been linked to increased IL-10 production after *in vitro* stimulation with PPD and further supports a link between IL-10 and anergic TST. It has also been shown that IL-10 is an important negative modulator of the DTH. Addition of anti-IL-10 antibodies prior to BCG vaccination and subsequent skin testing or the use of IL-$10^{-/-}$ mice both enhanced the IFNγ response to PPD and the swelling and duration of the DTH (Nadler, Luo et al. 2003). In addition, injection of recombinant IL-10 into the footpad of mice prior to challenge with antigen prevented the elicitation of contact hypersensitivity (CHS) in previously sensitized mice (Ferguson, Dube et al. 1994). These studies add further evidence that the skin test anergy observed in our study was regulated through an IL-10 dependent mechanism.

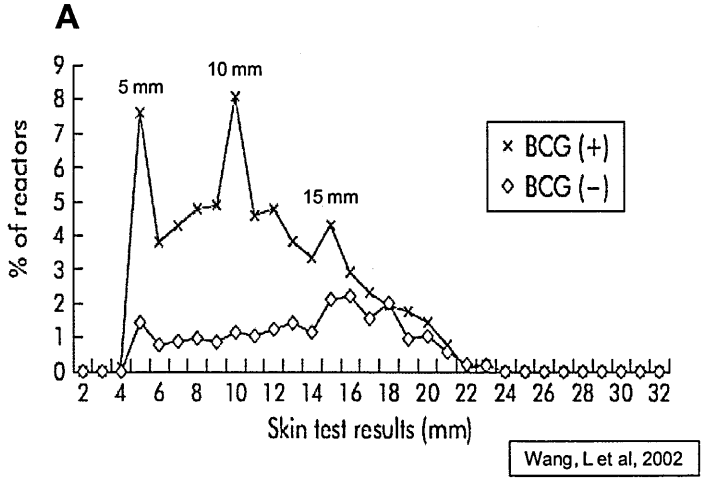
IFNγ production was higher in the TST responders but the concentration did not correlate with the induration. This discordance between IFNγ and TST induration may indicate that IFNγ is produced to initiate the DTH but the size of the induration that is recognised by increased swelling and recruitment of macrophages and T cells to the skin, is regulated by other factors. In support of this hypothesis, Martins *et al* investigated T cell function in relation to mycobacterial infections and found that in one subject, strong erythema to the TST without any induration, correlated with increased IFNγ response to PPD (Martins, Lima et al. 2007). In another study, mice with a disrupted IFNγ gene did not have a reduced DTH response compared to wild type (WT) but did have reduced

protection from TB in mice (Cooper, Dalton et al. 1993) indicating IFNγ is not the only pro-inflammatory molecule involved in initiating the DTH. Our results support the possibility that non-T cell derived IL-10 regulates the size of the induration. As mentioned before, cytokine production in the supernatant can be from many cell types. A study showed an association between $CD4^+CD69^+IFN\gamma^+$ among PBMCs cells and TST induration ($p = 0.04$) but this was not statistically significant when the TST was compared to IFNγ from culture supernatants ($p = 0.07$) (Martins, Lima et al. 2007). This is in keeping with the lack of correlation between ICS cytokines by flow cytometry and culture supernatants in our study. The TST results at 20 months indicated BCG vaccination at birth or 4½ months of age rarely caused a positive response and might be a better test of TB infection at this age. It would have been interesting to examine the correlation between IFNγ and IL-10 responses and TST in this age group.

It has been postulated that Tregs play a role in skin sensitivity to nickel allergy (Cavani, Nasorri et al. 2003). An infiltrate of Tregs possessing CLA occurred in anergic individuals at the patch site which constituted 20% of all the T cells at this site. When removing these Tregs in culture, T cells became more responsive to nickel. In contrast, Tregs from the blood of allergic individuals had limited or absent suppressive activity *in vitro* compared to Tregs from anergic subjects indicating the function of the Tregs was different (Cavani 2005). From our results $CD4^+CD25^+FOXP3^+$ Tregs were upregulated in response to PPD after BCG vaccination and were not different between responders and non-responders. This did not correspond to the nickel study although it is possible that the function of these Tregs is more impaired in the responders compared to the non-responders. The BCG vaccinated but TST anergic subjects exhibited reduced CD8 T cell proliferation in response to BCG, however T cell proliferation inversely correlated with the TST induration which is counter to what would have been expected. As with most immunological studies analysing peripheral blood, the homing of T cells to localised sites of disease may cause very different patterns to be seen at these sites that are not reflected

by changes in the peripheral blood. Previous studies in our lab observed reduced FOXP3 expression in peripheral blood from TB contacts (Burl, Hill et al. 2007) which in mice and monkeys has been shown to correspond to a homing of Tregs to the lung (Shafiana, S and Green, A *et al*, respectively, personal communication).

A peak of IFNγ reactivity at 10 mm induration corresponded to a peak percentage of skin test reactors at 10 mm. Figure 6.10A illustrates a previous study where three peaks were observed in BCG vaccinated individuals (5, 10 and 15 mm) (Wang, Turner et al. 2002). Our study also showed three distinct peaks at 7, 10 and 13 mm (Figure 6.10B). Wang *et al* did not discuss the relevance of these peaks in his study but they may represent different influences on the TST for example, exposure to NTM, BCG vaccination and *M.tb* latent infection or disease respectively. A mathematical approach to establishing 'cut offs' for LTBI was investigated by Villate *et al* which concluded that in 7 year old BCG vaccinated children there was a 97% probability of LTBI infection at an induration of > 17 mm and only 35% at > 10 mm and illustrated the distribution of induration that could be contributed to NTM, BCG and LTBI (Figure 6.10C)(Villate, Ibanez et al. 2006). Combined, these findings suggest that the TST reactivity is not a continuous variable, and therefore correlations between *in vitro* responses and TST induration may need to be evaluated differently.



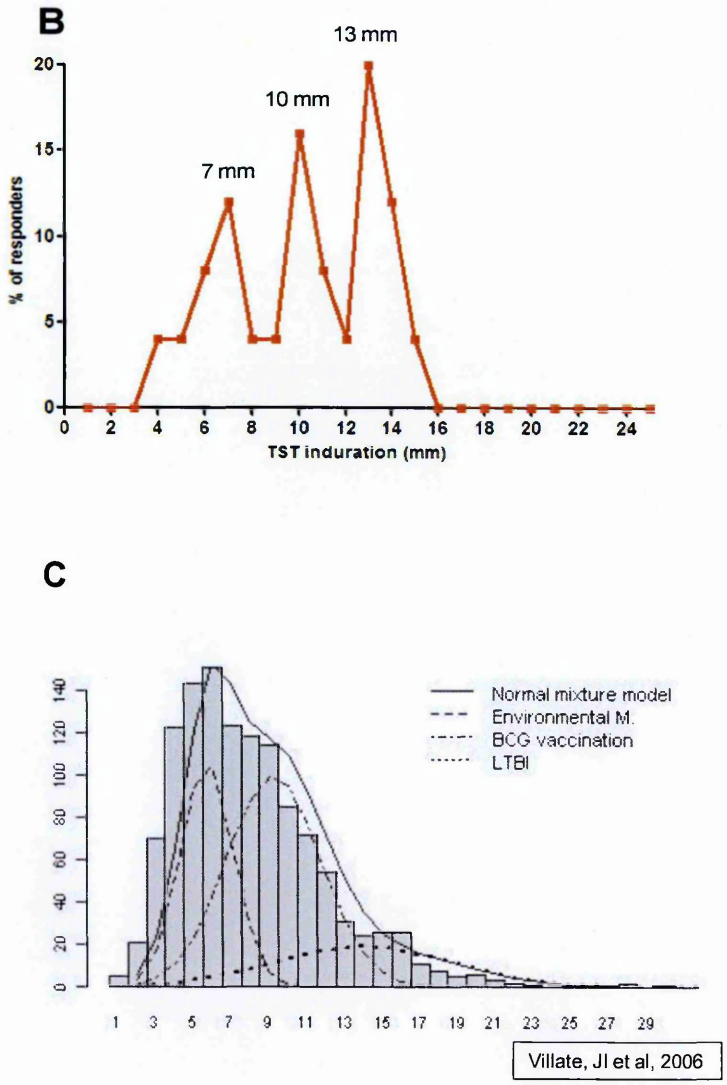


Figure 6.10: The relationship between frequency and TST induration. *(A) Results from a large cohort study illustrating three peaks of percentage of reactors at 5, 10 and 15 mm (Wang, L et al, 2002) using 2 T.U. RT23, (B) results from this study presented in a similar way to (A) and also illustrating three peaks at 7, 10 and 13 mm, using 2 T.U. PPD, (C) the observed induration distributions derived from a mathematical mixture model suggesting more accurate 'cut offs' for TST induration in 7 year old BCG vaccinated children (Villate, JI et al, 2006).*

There has been an extensive debate regarding the use of TST or the IGRA for diagnosing LTBI and TB disease. Largely, the specificity of the TST and the influence of

BCG vaccination have been questioned. Previous work in The Gambia suggested that the IGRA tests reflect load of *M.tb*, i.e. acute or recent infection, whereas TST indicated past and present infection (Hill, Fox et al. 2005) which may account for the lack of correlation. However Hill *et al* also found that in a Gambian setting the TST specificity was high with respect to sleeping proximity to the index case; sleeping closer to the index case was more likely to increase the reactivity to the TST (Hill, Brookes et al. 2006). The IGRA tests are changing current guidelines for diagnosing TB both in the US and throughout Europe, and may eventually replace the TST. However, amongst the many studies illustrating discordance between IFGA test and the TST, Fietta *et al* suggested that by increasing the stringency of the QuantiFERON-TB Gold 'cut off', there was correlation between the TST and the IFNγ production (Fietta, Meloni et al. 2003). Concordance between the TST and the IFNγ production was also found in patients with inflammatory bowel disease (Bartalesi, Vicidomini et al. 2008) indicating that evaluation in different clinical settings need to be assessed before discarding the use of the TST. A case contact study in The Gambia illustrated that of the contacts that progressed to disease, only 50% reacted to the TST or the IFNγ ESAT-6/ CFP10 ELISpot assays prior to disease but 71% were positive by one or other test suggesting positivity by either test might be the best indication for preventive treatment and would therefore not support the replacement of the TST in this setting (Hill, Jackson-Sillah et al. 2008). As a practical issue, a delay in processing the blood samples from cattle had a direct impact on the IGRA results from PPD stimulated samples (Whipple, Palmer et al. 2001; Gormley, Doyle et al. 2004). Although in an animal model, this suggests that the timing of blood collection may be crucial in detecting accurate results, which is important in many developing country settings. Furthermore, the IFNγ based tests are more expensive and technically demanding than the TST, which is an important consideration in resource-poor settings, where the burden of TB disease is often greatest. The use of ESAT-6 as a skin test has been developed recently and shown to be reactive in human subjects. Compared to PPD-tuberculin, 1μg ESAT-6 showed more

specific results producing reactivity in all TB contacts but not in BCG vaccinated individuals (Wu, Zhang et al. 2008) suggesting this antigen could be used in favour of the tuberculin skin test.

In summary our results showed that the positive TST observed at 4½ months of age is likely to be a result of BCG vaccination. This will confound the interpretation of the TST for the diagnosis of *M.tb* infection in early life but by 20 months of age the TST may be more reliable particularly if a higher cut off of 18 mm is used. Our results also suggest that BCG vaccination prior to exposure to NTM leads to less EC specific IL-10 compared to infants vaccinated on a background of NTM exposure which provides insights into the protective effects of BCG. Finally our results suggest that while IFNγ is induced by BCG vaccination, it is IL-10 that may control the size of the TST induration.

CHAPTER 7

Gender, CMV and non-specific reactivity

7.1 INTRODUCTION

BCG is a strong immune stimulator and has therefore been shown to have many non-specific effects (NSE) which have been exploited for therapeutic use. BCG can act as an adjuvant to boost the immune response that reduces tumour regression of various cancers, in particular bladder cancer where BCG has been used as treatment since 1976 (Morales, Eidinger et al. 1976). The mechanisms of this adjuvant effect are not clear but are thought to be related to Th1 cytokine production (e.g. IFNγ and TNFα) induced by the vaccine, recruiting and activating immune cells to fight the cancerous cells (Patard, Saint et al. 1998; Chen, O'Donnell et al. 2007) reviewed in (Herr and Morales 2008).

Given that BCG can induce a mature-like Th1 response in neonates (Marchant, Goetghebuer et al. 1999), it is therefore not surprising that recent reports have found NSE of BCG vaccination early in life. Given at birth, BCG has been found to reduce all cause mortality during infancy (Kristensen, Aaby et al. 2000; Breiman, Streatfield et al. 2004; Lehmann, Vail et al. 2005; Roth, Gustafson et al. 2005) and influence responses to other vaccines in early life (Ota, Vekemans et al. 2002). In an interesting infant study from Guinea Bissau, administrating BCG and OPV together in low birth weight children showed a reduction in immunogenicity to PPD antigen compared to BCG alone in these children (Peter Aaby, personal communication).

Interestingly some of these NSE appear to be gender specific (Aaby, Jensen et al. 2002; Elguero, Simondon et al. 2005; Moulton, Rahmathullah et al. 2005; Aaby, Ibrahim et al. 2006; Aaby, Jensen et al. 2006; Rodrigues, Fischer et al. 2006; Roth, Sodemann et al. 2006; Valentiner-Branth, Perch et al. 2007). Most of this work has been studied by Aaby *et al* in Guinea Bissau where they found that girls appeared to have lower all-cause mortality following BCG and measles vaccine (MV) but increased mortality following DTwP vaccine (Aaby, Jensen et al. 2002). This has also been shown in India (Moulton, Rahmathullah et al. 2005), Sudan and Democratic Republic of Congo (Aaby, Ibrahim et al. 2006). This work also suggested that the sequence of vaccines administered in early life

may have an influence on child mortality (Aaby, Ibrahim et al. 2006; Rodrigues, Fischer et al. 2006), and that it is the last vaccine given that dictated outcome (Aaby, Biai et al. 2007; Aaby, Garly et al. 2007). These NSEs are thought to be strongest in the 6 months following vaccine administration making our 4½ month sample time point of particular interest when looking for evidence of NSE.

Many animal and human studies have shown gender related dysfunction of the immune system. The incidences of many autoimmune diseases are biased towards females but often result in greater severity in males. With respect to infection, female mice have a greater Th1 response to many viruses thereby reducing the severity of some diseases but also increasing pathology in others (reviewed in (Shames 2002)). Immune cell phenotypes also differ between adult males and females whereby females have increased numbers of circulating CD4 T cells compared to males (Amadori, Zamarchi et al. 1995). In response to vaccination, female adult mice immunised with BCG showed higher levels of IFNγ than males (Huygen and Palfliet 1984). These gender differences are normally apparent post puberty and relate to sex hormones; androgens appear to have suppressive effects whereas oestrogens have pro-inflammatory effects. In addition, enhanced B cell function has been demonstrated in females which is mediated by oestrogen (Sthoeger, Chiorazzi et al. 1988).

Very few studies have examined gender related pre-pubescent differences but in some cases a reverse of the sex hormone difference is evident. A lack of sex hormones increases thymocyte development and increases the numbers of circulating immature T cells present in peripheral blood (reviewed in (Shames 2002)). Clear gender differences exist in the prevalence of asthma with a male preponderance before puberty and a reversal of this gender preference after adolescence (epidemiological review by (Chen, Mempel et al. 2008). This was also observed in the Gambian study of blood cell indices. The highest range for haemoglobin concentration was higher in females compared to males until 15 years of age where the reverse was true (Adetifa, Hill et al. 2008) indicating there are gender differences early in life that are not due to the influence of sex hormones.

Culturing conditions can also induce non-specific differences according to age. Culture of cord blood cells induced proliferation but the rate of apoptosis was also increased compared to adult cells which appeared to be independent of FasL and CD95 (Tu, Cheung et al. 2000; Yang, Hsu et al. 2001; Canto, Rodriguez-Sanchez et al. 2003) and neonatal cells have also been shown to proliferate strongly in response to nominal Ag largely by low affinity promiscuous TCR/MHC interactions (Gavin and Bevan 1995; Le Campion, Lucas et al. 2002), suggesting possible mechanistic differences between cord blood and infant peripheral blood that could influence their responses to specific stimuli.

Immunological studies in The Gambia have shown that CMV infection in early life has a profound effect on the immune system, decreasing cell-mediated immune responses and altering the phenotype of CMV specific and non-specific CD8 T cells to a more differentiated state (Miles, van der Sande et al. 2007; Miles, Sanneh et al. 2008). Measles specific antibodies and CD4 IFNγ responses were also reduced in CMV infected children post measles vaccination suggesting that CMV may affect immunological memory to vaccines (Miles, van der Sande et al. 2008). A striking 90% of children in The Gambia are infected with CMV by one year of age, and approximately 4% are infected intrapartum or during delivery (Miles, van der Sande et al. 2007). This contrasts to a middle-upper socioeconomic setting where 50 – 80% of adults are infected by 40 years of age (www.cdc.gov/cmv/facts.htm). Clinical evidence has shown hearing and visual loss, mental retardation and sometimes death in affected newborns (reviewed in (Hassan and Connell 2007)). It was therefore important to examine the impact of CMV infection on responses to the BCG vaccination in our study.

Non-specific effects of BCG vaccination were assessed in our study by examining the responses to a superantigen, *Staphylococcal Enterotoxin B* (SEB) that also acted as a useful positive control in our study. Superantigens bind to Major Histocompatibility Complex (MHC) class II molecules on antigen presenting cells (APCs) without the need

for peptide processing, and then form a complex with a TCR that possesses a homologous Vβ chain (Hewitt, Lamb et al. 1992). They are thus able to elicit a non-specific T cell response.

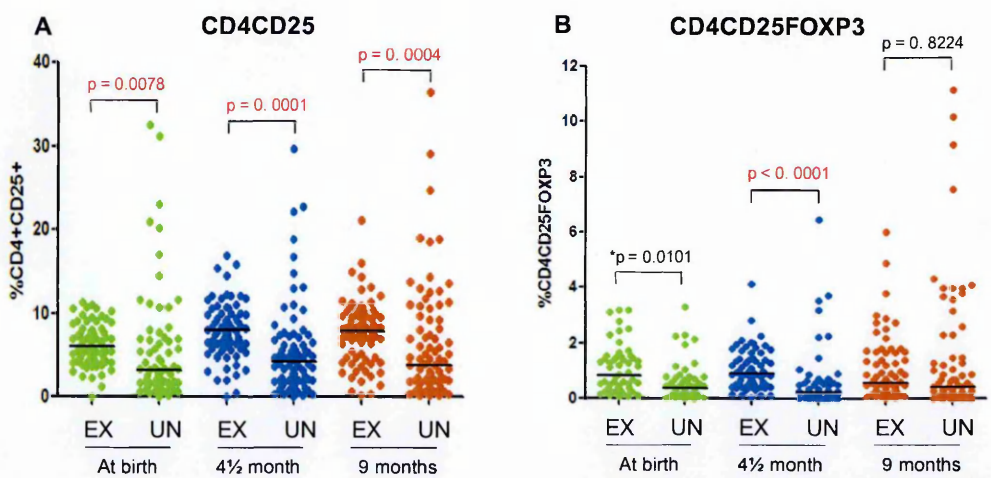
This chapter analyses the non-specific effects of culture according to age, and then analyses for the effect of BCG vaccination status on responses to SEB. Analysis of gender effects are also shown in relation to specific and non-specific stimuli and BCG vaccination group. Finally, the effect of CMV infection was considered on the various parameters analysed in this study.

7.2 RESULTS

7.2.1 Age specific differences in T cell turnover and apoptosis during culture

In the *ex vivo* studies only a proportion of the 100 µL sample of whole blood was required to acquire 500,000 events on the flow cytometer, but after 5 days of culture the entire sample (which equated to 140µl of original whole blood) was acquired and the average number of events acquired was often < 200,000 suggesting a large amount of cell death during culture.

To understand the effect of cell culturing on the distribution of various T cell subsets after 5 days of culture, the proportions of T cells in unstimulated cultures was compared to those present *ex vivo,* in the freshly stained whole blood. The lymphocyte population and the proportions of $CD8^+$ T cells, $CD4^+CD25^+$ T cells and $CD4^+CD25^+FOXP3^+$ Tregs in cord blood and at 4½ months of age were reduced significantly in culture compared to *ex vivo*. By 9 months of age culturing had less of an effect on the T cell proportions and only the $CD4^+CD25^+$ population was reduced (Figure 7.1A and B). An increase in $CD4^+$ T cells was observed at 4½- and 9- months of age after culture but not in cord blood, an effect likely to be due to the high rate of $CD4^+$ T cell apoptosis at birth (Figure 7.1C).



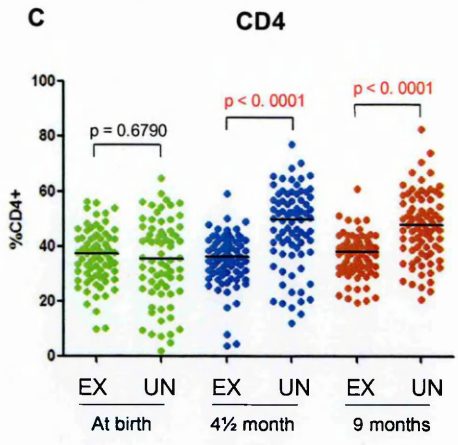


Figure 7.1: Comparisons of T cell phenotypes pre- and post- 5 days of culture. *100 µL whole blood was phenotyped straight after collection (ex vivo) and compared to in vitro culture for 5 days without antigen. A Wilcoxon non-parametric paired test was applied at 5% significance. Proportions of $CD4^+CD25^+$ (A), $CD4^+CD25^+FOXP3^+$ (B) and $CD4^+$ T cells populations were plotted for each time point, median values are represented as black lines, at birth n = 78 , at 4½ months n = 79 and at 9 months n = 84, * not significant after Bonferroni correction for multiple testing.*

To further analyse the differences observed in culture with age, longitudinal comparisons were analysed. Most differences were apparent when comparing cord blood and 4½ months, whereas T cell frequencies in unstimulated cultures were often comparable at 4½- and 9 months of age. Lymphocyte populations (proportion of lymphocytes and absolute numbers) were lower in day 5 cord blood (3.36%) compared to infant blood (37.96%) (Figure 7.2A - C), and there was a high proportion of cellular debris (probably to be due to dead cells) in cord blood which likely contributed to the reduced proportion of lymphocytes among total events acquired (green oval in Figure 7.2A and B) in comparison to lymphocytes from *ex vivo* phenotyping (Figure 7.2D).

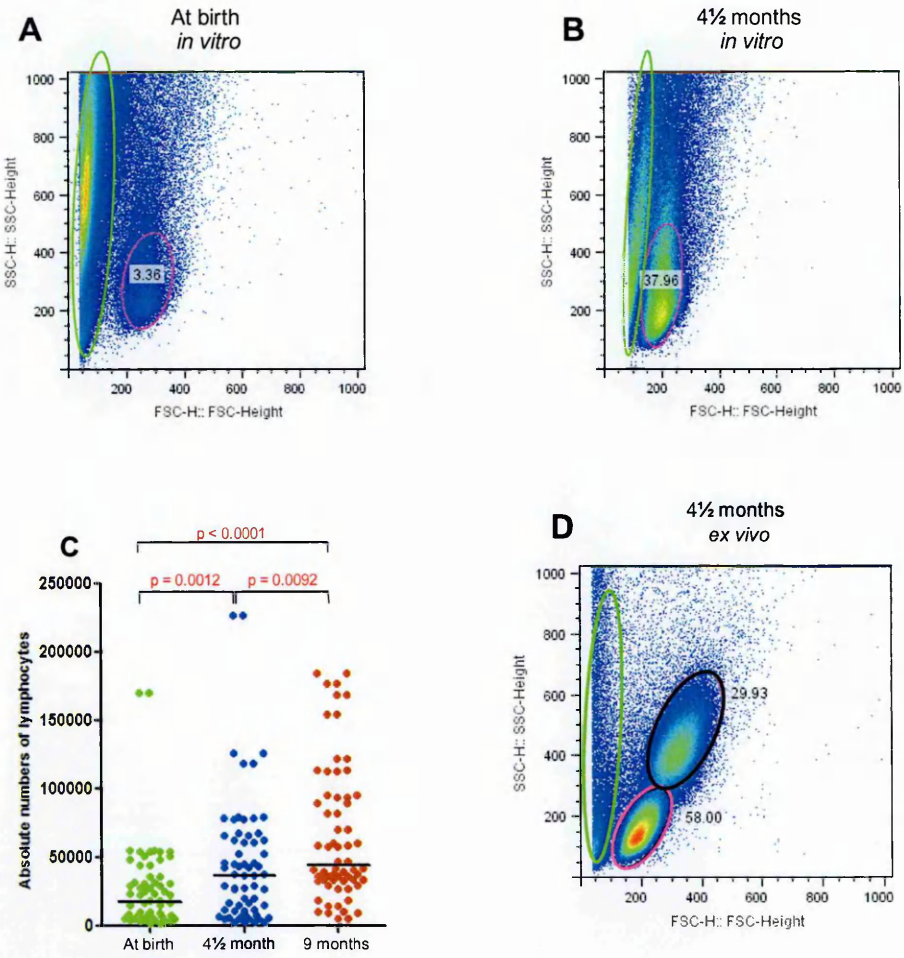
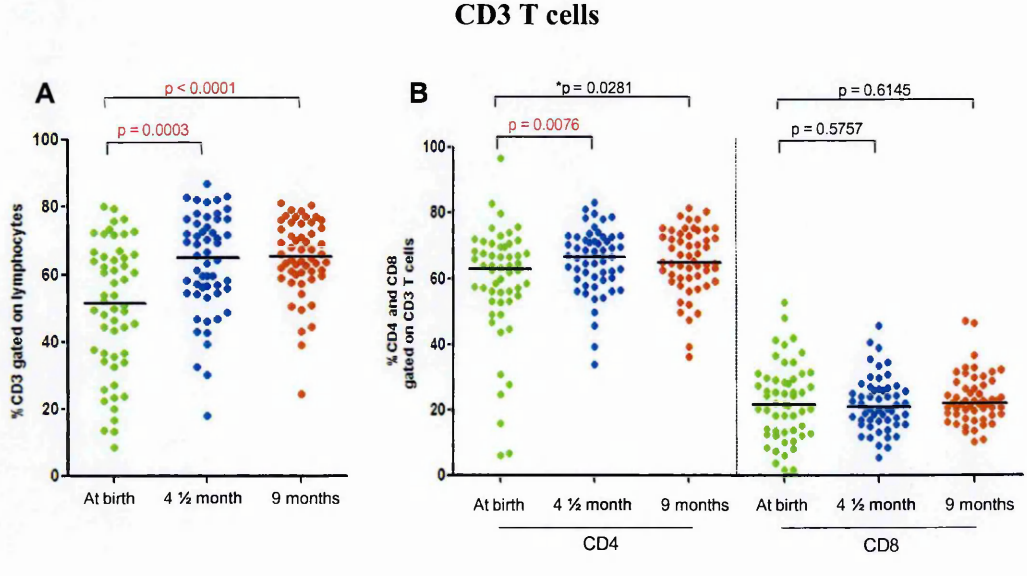


Figure 7.2: Lymphocyte populations after in vitro culture for 5 days. *500 µL cord blood and peripheral blood at 4½ months of age were diluted 1: 5 and cultured for 5 days. The profile of T cells in unstimulated cultures at birth (A), at 4½ months of age (B) and 4½ months ex vivo with 100 µL whole blood (D) were compared, pink oval represents the lymphocyte population, green oval represents cellular debris, black oval represents a larger and more granular population (possibly macrophages). FSC = refers to size of event acquired, SSC = refers to the density or granularity of the event acquired by flow cytometry. Absolute numbers of lymphocytes in unstimulated cultures were compared across each age group (C). A Wilcoxon non-parametric paired test was used at 5% significance, the black bar represents the median value, n = 64.*

The reduced number of lymphocytes observed in cord blood corresponded to a

reduction in percentage of $CD3^+$ T cells (median 51.41%) in this population compared to 4½- (median 64.95%) and 9- months (median 65.26%) of age (Figure 7.3A). This reduction was likely to be due to the $CD4^+$ population since the proportion of $CD8^+$ T cells within the $CD3^+$ T cell subset did not change between age groups but the $CD4^+$ T cells were reduced (Figure 7.3B). Cord blood also had increased proliferation of lymphocytes, as measured by Ki-67 expression, compared to the later time points (Figure 7.3C). Only $CD8^+$ cells had significantly higher proliferation at birth compared to later so these are most likely responsible for the observation (Figure 7.3D). IL-10 production was assessed intracellularly by flow cytometry and also in the supernatant of the 5 day cultures. There were less IL-10^+ lymphocytes in cord blood compared to infants with significant upregulation in $CD8^+$ T cells but not $CD4^+$ T cells after birth (Figure 7.3E and F). However the differences observed in $CD8^+$ T cells were no longer significant after Bonferroni correction factor was applied.



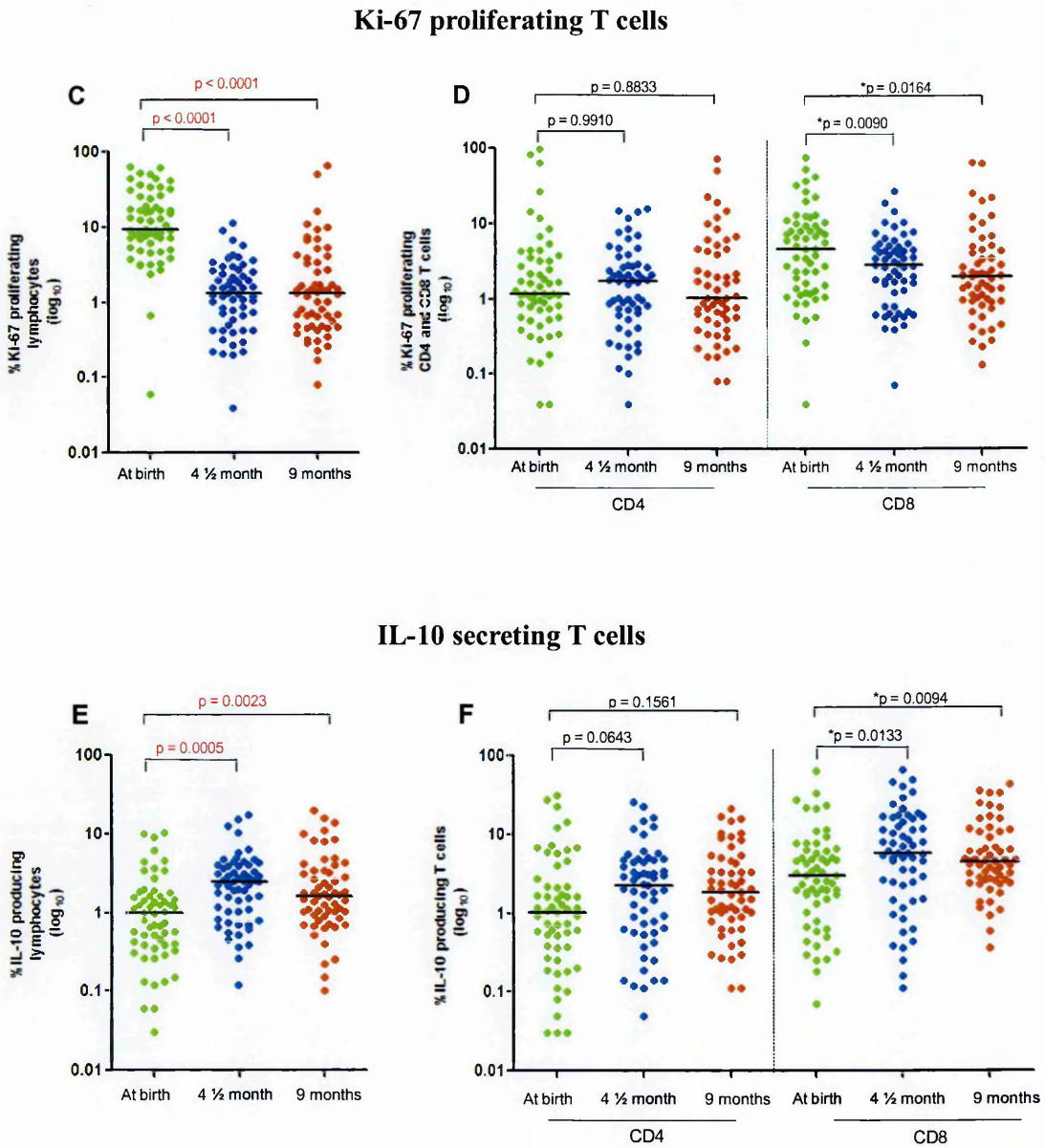


Figure 7.3: T cell populations in unstimulated cultures. *500 µL cord blood and peripheral blood at 4½- and 9- months of age were diluted 1: 5 and cultured for 5 days without antigen. The profile of T cells in unstimulated cultures was compared. The proportion of lymphocytes that were $CD3^+$ (A), $CD3^+CD4^+/CD8^+$ (B), $Ki\text{-}67^+$ (C), $CD4^+/CD8^+Ki\text{-}67^+$ (D), $IL\text{-}10^+$ (E) and $CD4^+/CD8^+IL\text{-}10^+$ T cells were compared across all three age groups. A Wilcoxon non-parametric paired test was used at 5% significance, the black bar represents the median value, * not significant after Bonferroni correction for multiple testing, n = 64.*

In contrast, there were no significant differences in the production of IL-10 in the supernatant of unstimulated cultures between the age groups with moderate production at all ages (Table 7.1). With respect to IFNγ and IL-13 cytokine production, they were both lower at birth than at 4½ months of age. Cytokine production was often low in unstimulated samples but they were generally within the dynamic ranges described by the commercial supplier of the assay kits (IFNγ, 2.14 – 31,000 pg/mL; IL-10, 1.6 – 26,000 pg/mL; IL-13, 0.56 – 9,000 pg/mL) and therefore the associations within different age groups could be considered genuine.

	At birth pg/ml (range)	4½ m pg/ml (range)	9 m pg/ml (range)	At birth vs 4½ m	At birth vs 9 m	4½ m vs 9 m
IFNγ	0 (0 – 444.5)	45.92 (0 – 1,575)	7.25 (0 – 19,819)	< 0.0001	0.0001	0.0739
IL-10	4.87 (0.7 – 536.8)	3.5 (0 – 288.1)	5.29 (0.7 – 624.1)	0.0843	0.0958	0.4989
IL-13	0 (0 – 9.98)	2.33 (0 – 368.4)	1.96 (0 – 854.7)	< 0.0001	< 0.0001	0.6273

Blue text = increased cytokine production compared to the earlier age group

Table 7.1: Cytokine production in unstimulated culture conditions. *500 μL cord blood and peripheral blood at 4½- and 9- months of age were diluted 1: 5 and cultured for 5 days without antigen. Comparisons of cytokine production (pg/mL) between age groups in unstimulated 5 day whole blood cultures. A non parametric Wilcoxon paired test at 5% significance was used to analyse the data, n = 73.*

A larger proportion of lymphocytes contained intracellular TGFβ compared to IL-10 after 5 days of culture, particularly among $CD4^+$ T cells in cord blood. This might be because $TGF\beta^+$ T cells are more resistant to apoptosis or cord blood $CD4^+$ T cells are genuinely increased and have a suppressive function. When correcting for multiple testing

the TGFβ and CD8 specific differences were no longer significant.

Interestingly the numbers of activated T cells ($CD4^+CD25^+$) and $FOXP3^+$Tregs ($CD4^+CD25^+FOXP3^+$) in culture were unaltered across all ages.

A summary of the comparisons between cord blood and 4½ months of life in unstimulated cultures is found in Table 7.2.

Unstimulated cultures	Cord blood and 4½ months of age
Lymphocytes	+
CD3	+
CD3(CD4)	*+
CD3(CD8)	=
CD4(CD25)	=
CD4(CD25FOXP3)	=
Ki-67	-
CD4(Ki-67)	=
CD8(Ki-67)	*-
IL-10	+
CD4(IL-10)	=
CD8(IL-10)	*+
TGFβ	*-
CD3CD4(TGFβ)	*-
CD3CD8(TGFβ)	=
IFNγ cytokine	+
IL-10 cytokine	=
IL-13 cytokine	+

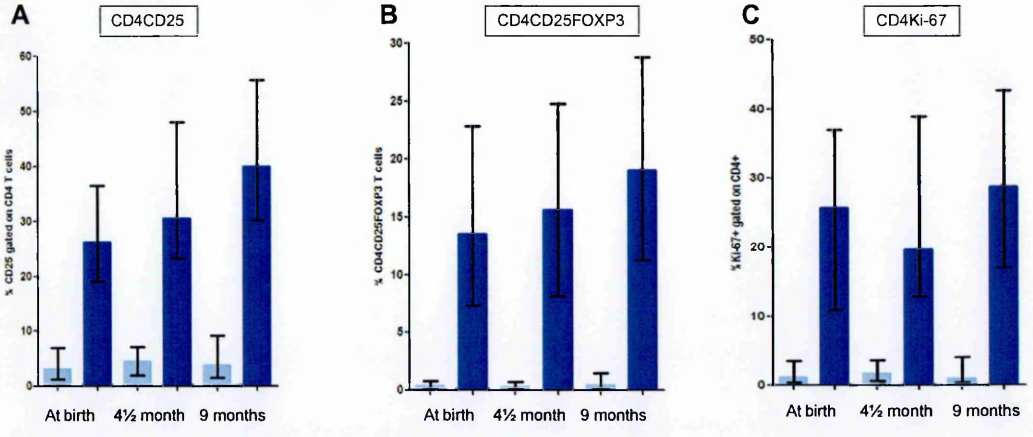
Table 7.2: Summary of differences in cord blood immune populations in unstimulated cultures compared to 4½ months. *500 μL cord blood and peripheral blood at 4½ months of age were diluted 1: 5 and cultured for 5 days without antigen. The profile of T cells (%) and cytokine production (pg/ml) in unstimulated cultures was compared between cord blood and 4½ months of age, '+' and dark blue represents higher levels at 4½ months than in cord blood, '-' and light blue represents lower levels at 4½ months than in cord blood, '=' and no highlighted background represents similar levels at 4½ months and in cord*

*blood. A Wilcoxon non-parametric paired test was used at 5% significance, *not significant after Bonferroni correction for multiple testing, n = 64.*

Analysis of unstimulated data with respect to the BCG vaccine schedule at all time points did not reveal any differences, suggesting that BCG did not influence the immune cells in unstimulated cultures. This further indicates that differences in the antigen-stimulated cultures are genuine specific effects.

7.2.2 Responses to SEB

At 4½- and 9- months of age almost all the parameters were increased in response to SEB compared to background, with the exception of $CD3^+CD4^+$ T cells that were downregulation. Thus, $CD3^+$ T cells, $CD8^+$ T cells, $CD4^+CD25^+$, $CD4^+CD25^+FOXP3^+$, Ki-67 (both $CD4^+$ and $CD8^+$), intracellular IL-10 (both $CD4^+$ and $CD8^+$) and intracellular TGFβ (both $CD4^+$ and $CD8^+$) and supernatant IFNγ, IL-10, IL-13 were all significantly higher than in unstimulated cultures even after correcting for multiple testing (Figure 7.4 A - E). The magnitude of responses to SEB in cord blood was comparable to 4½ and 9-months of age with the exception of no upregulation of IL-10 production from $CD8^+$ T cells (Figure 7.4D pink square, p = 0.7311) or TGFβ producing T cells (Figure 7.4E pink square, p = 0.3255) above background unstimulated levels (Figure 7.4A - E).



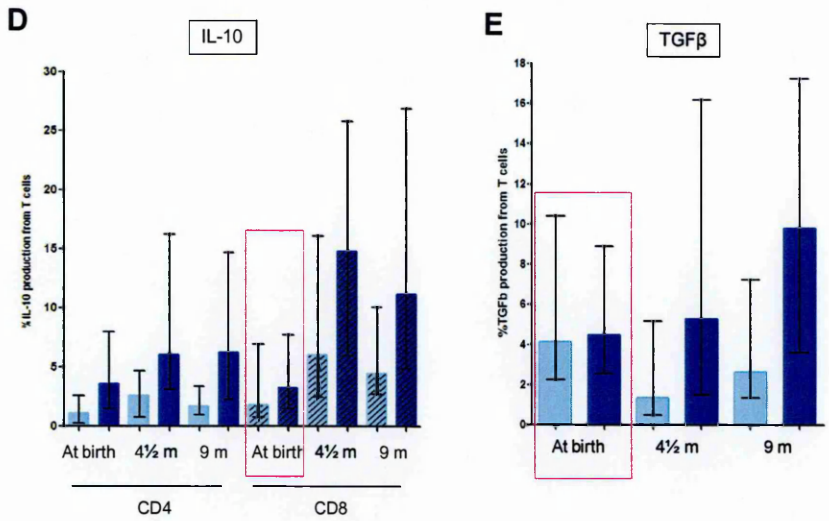


Figure 7.4: In vitro T cell responses to SEB superantigen. *500 µL cord blood and peripheral blood at 4½- and 9- months of age were diluted 1: 5 and cultured for 5 days with SEB. The profile of $CD4^+CD25^+$ (A), $CD4^+CD25^+FOXP3^+$ (B), $CD4^+Ki\text{-}67^+$ (C), IL-10 produced by T cells (D) and TGFβ produced by T cells (E) were compared in unstimulated (pale blue) compared to SEB stimulated (dark blue) cultures across all three age groups. Pink square represents the lack of SEB induced response compared to unstimulated for the cord blood. A Wilcoxon non-parametric paired test was used at 5% significance, bars represent mean values with SEM, at birth n =70, 4½ months n = 76 and 9 months n = 80.*

After subtracting the unstimulated values and comparing across age groups, there was an increased frequency of activated T cells and overall proliferating cells but also greater Th2 (IL-13) and suppressor T cell responses (IL-10, TGFβ) to SEB at 4½- and 9-months of age compared to cord blood, but similar Th1 (IFNγ) and T cell proliferation (Ki-67) across all ages (Table 7.3). In particular IL-10 and IL-13 production in culture supernatants were increased at 9 months compared to 4½ months (Table 7.3). In addition, the level of $FOXP3^+$Tregs was similar across all age groups suggesting that the ability to induce Tregs in response to a strong polyclonal stimulus is present and stable from birth

although the function of these Tregs cannot be elucidated from our results (Figure 7.4B and Table 7.3).

	At birth	4½ months	9 months	At birth vs 4½ m	At birth vs 9 m	4½ vs 9 m
CD3	-1.385	6.77	8.295	0.0669	*0.0252	0.5238
CD4	-2.395	-1.66	-0.9	0.9051	0.3541	0.7631
CD8	2.7	4.43	6.225	0.0593	*0.0252	0.1567
CD4(CD25)	22.32	25.64	33.5	*0.0386	< 0.0001	0.1409
CD4(CD25FOXP3)	13.53	12.86	17.50	0.7191	0.2456	0.6794
Ki-67	9.58	14.22	18.5	*0.0484	0.0019	0.3774
CD4(Ki-67)	16.58	16.92	21.89	0.5055	0.1420	0.2908
CD8(Ki-67)	13.95	15.61	18.64	0.6816	0.2726	0.3137
IL-10	0.28	5.565	2.475	< 0.0001	*0.0058	*0.0238
CD4(IL-10)	0.465	1.05	3.725	0.0025	*0.0108	0.8067
CD8(IL-10)	-0.145	7.55	6.415	< 0.0001	0.0001	0.7166
TGFβ	0.42	0.44	4.285	0.1734	0.0006	0.2646
CD3CD4(TGFβ)	0.395	0.58	5.045	0.4917	*0.0055	0.2206
CD3CD8(TGFβ)	0.54	2.3	7.805	*0.0292	0.0016	0.4525
IFNγ cytokine	7672	9039	7101	0.1416	0.5567	0.411
IL-10 cytokine	148.1	363.1	546.6	< 0.0001	< 0.0001	0.0003
IL-13 cytokine	355.3	1860	2867	< 0.0001	< 0.0001	0.0007

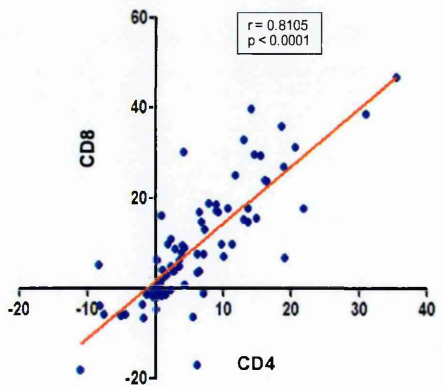
Blue text = increased cytokine production compared to the earlier age group

Table 7.3: Longitudinal responses to SEB. *500 µL cord blood and peripheral blood at 4½- and 9- months of age were diluted 1: 5 and cultured for 5 days with SEB. The profile of T cells (%) and cytokine production (pg/ml) was compared across age groups after subtracting the background unstimulated responses. A Wilcoxon non-parametric paired test was used at 5% significance, at birth n =70, 4½ months n = 76 and 9 months n = 80.*

SEB stimulation induced IL-10 production by both $CD4^+$ and $CD8^+$ T cells in early life. A paired correlation of IL-10 produced by $CD4^+$ and $CD8^+$ T cells at 9 months showed that those individuals with a high percentage of IL-10 producing CD4 T cells, also had a high percentage of IL-10 producing CD8 cells (Figure 7.5A). The same pattern occurred for cord blood and at 4½ months. Generally there was a higher percentage of CD8 cells

producing IL-10 in response to SEB, although this difference was only significant at 9 months (Figure 7.5B). Indeed, in cord blood the reverse was true and T cell production of IL-10 was predominantly from CD4 T cells (Figure 7.5B).

A



B

IL-10	CD4	CD8	*p value*
At birth	0.52	-0.15	<0.0001
4½ months	3.53	5.93	0.2255
9 months	3.83	6.42	0.0003

Figure 7.5: IL-10 production from T cells in response to SEB. *Whole blood was cultured for 5 days with SEB and a comparison of T cell production of IL-10 was assessed at 4½ months and 9 months. Correlation between CD4 and CD8 production of IL-10 at 9 months of age (A) illustrating a positive correlation (Spearman's correlation coefficient r = 0.8105, p< 0.0001). (B) Comparison between percentage of $CD4^+$ and $CD8^+$ T cells that produced IL-10 after SEB stimulation at birth, 4½- and 9- months of age. A Wilcoxon non-parametric paired test was applied at 5% significance, at birth n = 76, at 4½ months n = 77, 9 months n = 80. Median values of unstimulated values subtracted from SEB stimulated values are presented. Pale blue text indicates a significant upregulation in CD8*

T cells compared to CD4 T cells and red text indicates a significant downregulation in CD8 T cells compared to CD4 T cells.

Analysing the SEB responses by vaccination group showed no significant differences at any of the age groups for any of the parameters studied. In particular there were no differences between vaccinated and unvaccinated children at 4½ months of age after adjustment for multiple correction, although there was a borderline increase in IL-10 production in the culture supernatants in the BCG naïve group at 4½ months but this was not significant after adjustment for multiple testing (p = 0.0421). Figure 7.6 illustrates the cytokine profiles in response to SEB according to age and BCG vaccination schedule.

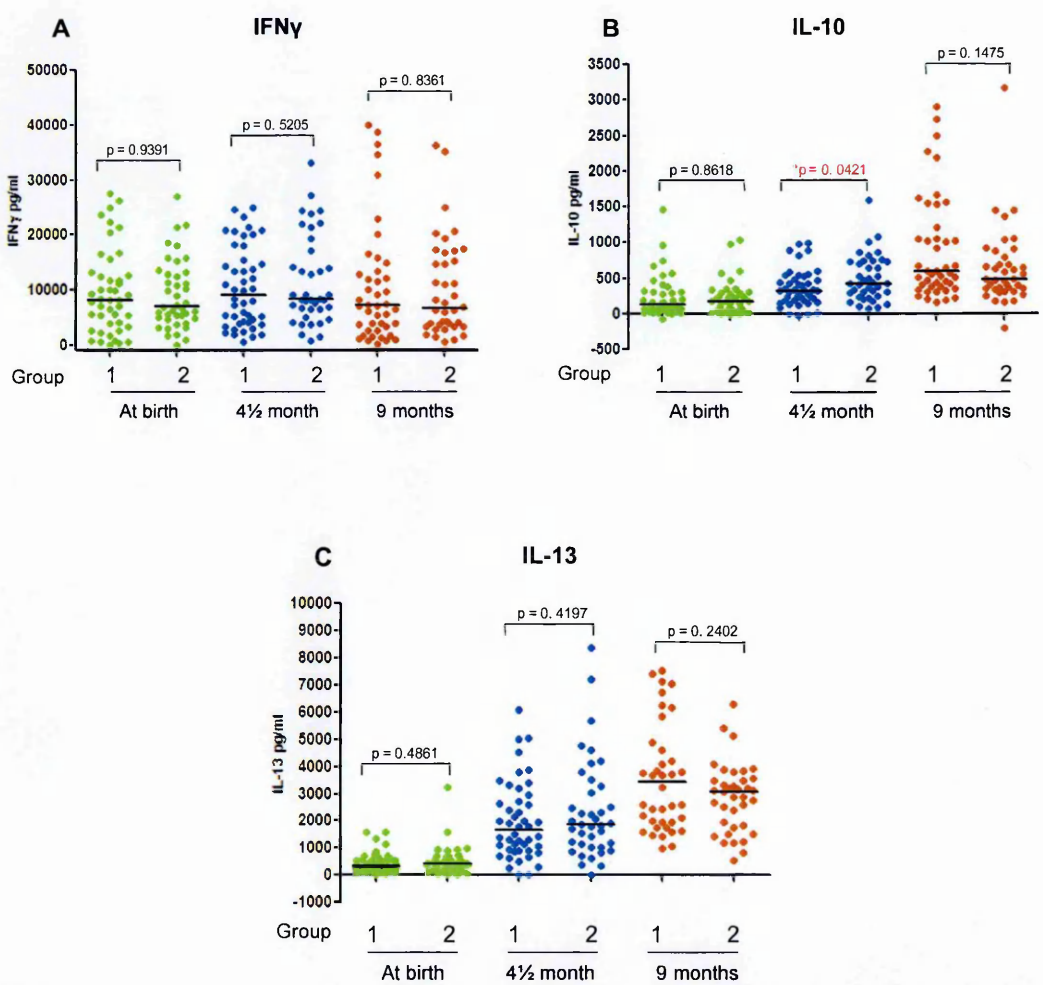


Figure 7.6: Effect of BCG vaccination on SEB responses. *500 μL cord blood and peripheral blood at 4½- and 9- months of age were diluted 1: 5 and cultured for 5 days with SEB. The supernatant cytokine profile of (A) IFNγ, (B) IL-10 and (C) IL-13 was compared between Group 1 (vaccinated at birth) and Group 2 (vaccinated at 4½ months) at each time point. A Mann Whitney U test was applied to compared between groups at 5% significance, the black bar represents the median value, * not significant after Bonferroni correction for multiple testing, at birth Group 1 n = 45 Group 2 n =42 , at 4½ months Group 1 n = 46 Group 2 n = 38 , 9 months Group 1 n =45 Group 2 n = 40.*

7.2.3 CMV infection

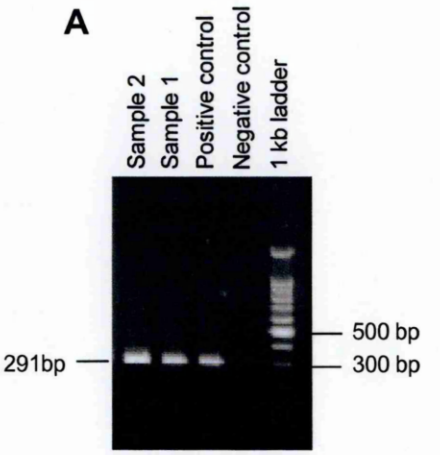
Urine samples were analysed for cytomegalovirus (CMV) infection on the day of bleeding as a surrogate of infection. The CMV nested PCR assay identified a 291kb product from the membrane-associated protein, UL50 sequence of human herpes virus 5 in the urine (Figure 7.7A). Congenital infection was defined as infection within the first two weeks of life. Overall there were 16.0% congenitally infected neonates which was approximately10-fold higher than the global average (Hassan and Connell 2007). By 4½ months of age 47.1% of infants were infected and by 9 months of age there was 63.1% infected infants (Figure 7.7B).

Infants and children often shed virus for years making it difficult to estimate the time of infection when evaluating one time point. In our study persistent shedding was not always observed. Thirteen subjects exhibited intermittent shedding over the 9 months (Figure 7.7C). These results may indicate that shedding of the virus was not continuous but may also indicate that the amount of virus was at the level of detection using our PCR assay.

After segregating according to vaccine status the percentage of positive individuals was similar in each group at each time point (Fisher's exact test; at birth p = 1.0, 4½ months p = 0.8284, 9 months p = 0.5005) indicating that the BCG vaccine delay did not

affect CMV infection status (Figure 7.7D and E).

A



B

	At birth	4½ months	9 months
positive	15	40	53
total	94	85	84
% positive	15.96	47.06	63.10

C

At birth	4½ months	9 months	Number of subjects (group 1, group 2)
+	-	+	4 (2, 2)
-	+	-	4 (3, 1)
+	+	-	1 (0, 1)
+	-	-	3 (1, 2)
+	-	ND	1 (1, 0)

D

	At birth		4½ months		9 months	
Group	**1**	**2**	**1**	**2**	**1**	**2**
positive	8	7	22	18	26	28
total	51	43	45	40	43	41
% positive	15.7	16.2	48.9	45.0	60.5	68.3

E

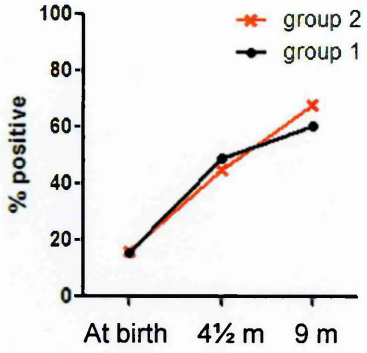


Figure 7.7: CMV infection status. *A nested PCR assay amplified a 291bp fragment of CMV DNA in the urine of CMV infected subjects (A). Numbers of infected children at each time point were identified and % positivity (B), numbers of children with discordant infection status over 9 months (C) and numbers of children in each group at each time point were calculated (D). A graphical comparison of CMV infection over time between Group 1 and Group 2 (E).*

Ex vivo $CD8^+$ T cell populations are increased in CMV infected individuals

CMV frequently causes a profound expansion of CMV specific $CD8^+$ T cells (Marchant, Appay et al. 2003; Gibson, Piccinini et al. 2004; Miles, van der Sande et al. 2007; Miles, van der Sande et al. 2008; Chidrawar, Khan et al. 2009). Thus it was not surprising that CMV infected children had a greater proportion of $CD8^+$ T cells *ex vivo* at both 4½- and 9- months of age, irrespective of vaccine group (Figure 7.8A). Interestingly this effect was not observed in those congenitally infected at birth although the number of CMV infected neonates is very small (n = 5). There was a borderline significant decrease in *ex vivo* $CD4^+$ T cells at 4½ months in the CMV infected infants ($p = 0.0459$) but this was eliminated after correction for multiple testing. $CD4^+$ T cells were similar across the age groups in the uninfected infants (Figure 7.8B).

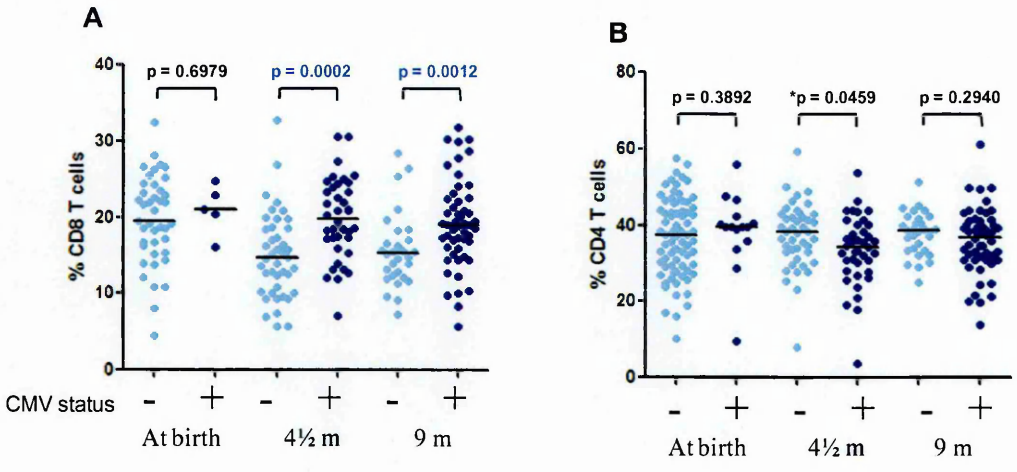


Figure 7.8: Ex vivo T cell populations affected by CMV infection over time. *100 µL whole blood was collected and immediately phenotyped (ex vivo). The proportions of lymphocytes that were $CD8^+$ (A) and $CD4^+$ (B) were analysed according to CMV status across all age groups irrespective of BCG vaccine schedule. A Mann Whitney U test was applied at 5% significance, the black bar represents the median value, *not significant after Bonferroni correction for multiple testing. At birth CMVneg n = 76 CMVpos n = 15 (CD8: CMVneg n = 41 CMVpos n = 5), at 4½ months CMVneg = 42 CMVpos = 38, 9 months CMVneg = 30 CMVpos = 52.*

The increase in $CD8^+$ T cells observed in the CMV infected infants resulted in a lower ratio of CD4: CD8 T cells among the infected infants at 4½- ($p < 0.0001$) and at 9-months ($p = 0.0043$) compared to the uninfected individuals. CD4: CD8 ratios of < 1 are considered an 'immune risk phenotype' which is well described in relation to chronic CMV infection (reviewed in (Koch, Solana et al. 2006). At 4½- and 9- months of age, 8% and 10% respectively of CMV infected infants had a CD4:CD8 ratio < 1 compared to 2% and 3% in the uninfected infants at the same time points. No other parameter studied *ex vivo* was affected by CMV status. In addition these findings were not altered by the different BCG vaccination schedules of the two groups.

CMV infection altered superantigen and anti-mycobacterial immunity

Miles *et al* have shown enhanced $CD8^+$ T cell responses to SEB in infants with CMV in the same population as our study (Miles, Sanneh et al. 2008). Similarly in our study increased IFNγ production was observed in response to SEB in cord blood of congenitally infected individuals (n = 14) compared to uninfected infants (p = 0.0162) (Figure 7.9A). However $CD4^+CD25^+$ activated T cells were significantly reduced in response to SEB in the cord blood of CMV infected neonates (p = 0.0024) (Figure 7.9B). No other responses to SEB were altered according to CMV infection, including $CD4^+CD25^+FOXP3^+$ Tregs and $CD8^+$ T cells. Cord blood responses to mycobacterial antigens, (PPD, BCG and EC) were unaffected by CMV infection status (data not shown).

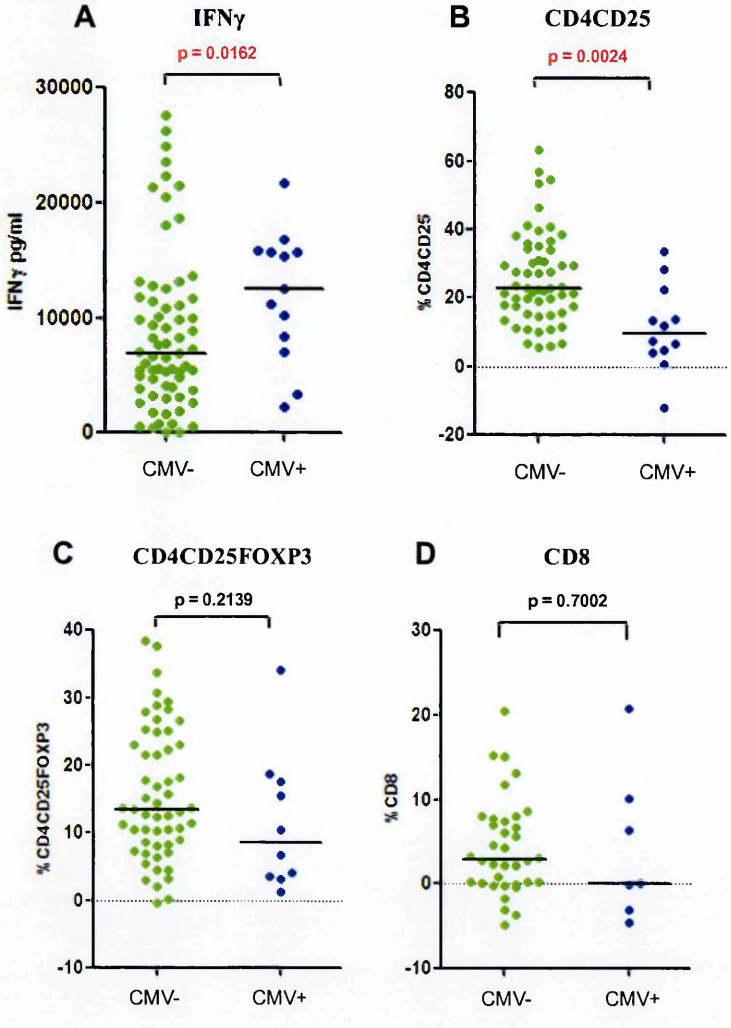


Figure 7.9: Congenital CMV infection alters cord blood responses to SEB. *500 μL cord blood was diluted 1: 5 and cultured for 5 days with SEB. IFNγ cytokine production in the supernatant of cultures (A), $CD4^+CD25^+$ (B), $CD4^+CD25^+FOXP3^+$ (C) and $CD8^+$ (D) T cells were compared according to CMV status. A Mann Whitney U test was applied to compared between groups at 5% significance, the black bar represents the median value, CMVneg n =55 (CD8: n = 36) , CMVpos n =12 (CD8: n = 7) .*

At 4½- and 9- months of age, CMV infection had little effect on the immune response to SEB and mycobacterial antigens. At 4½ months SEB stimulated CD8 T cells were greater in CMV infected individuals (p = 0.0093), although not significant after correction for multiple testing (Figure 7.10A). Responses to PPD are usually stronger than to other mycobacterial antigens, however most CMV related effects were observed in response to BCG. The frequency of BCG stimulated $CD4^+$ and $CD8^+$ IL-10 producing T cells were reduced in the CMV infected individuals at 4½ months (CD4 p = 0.0126, CD8 p = 0.0270) and at 9 months of age (CD4 p = 0.0281, CD8 p = 0.0419) although not significant after correction for multiple testing (Figure 7.10B and C). However there were no significant changes in IL-10 production from the supernatants of cultures stimulated with SEB or mycobacterial antigens (data not shown). After adjusting for multiple testing there were no CMV related differences within BCG vaccine groups at 4½- or 9- months of age.

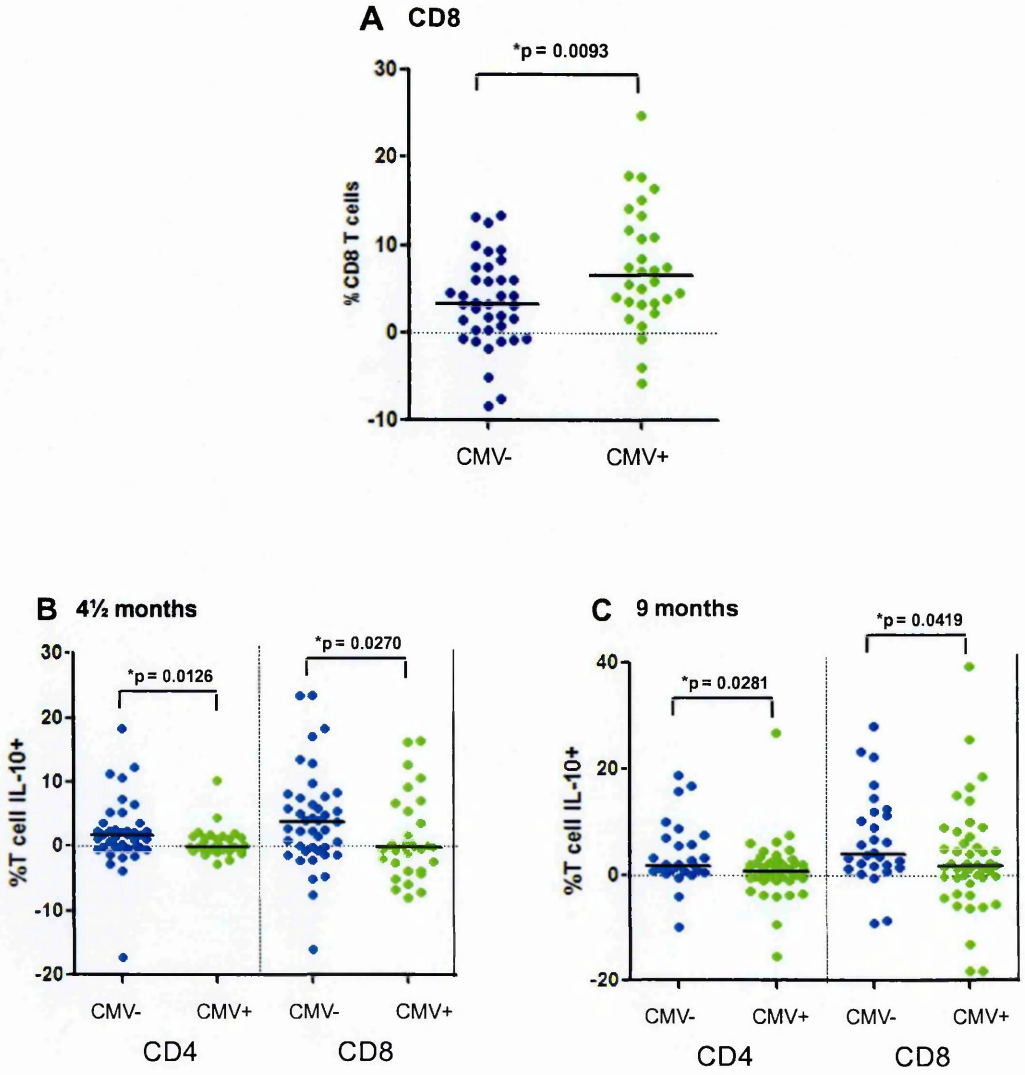


Figure 7.10: Responses to SEB and BCG in CMV infected infants at 4½ and 9 months of age. *500 µL peripheral blood was diluted 1: 5 and cultured for 5 days SEB (A) and BCG (B and C). $CD8^+$ (A), $CD4^+$ $IL\text{-}10^+$ (B), $CD8^+$ $IL\text{-}10^+$ (C) T cells were compared according to CMV status at 4½ months of age (A and B) and 9 months of age (C). A Mann Whitney U test was applied to compared between groups at 5% significance, the black bar represents the median value, * not significant after Bonferroni correction for multiple testing, 4½ months, CMVneg n =43 , CMVpos n =33; 9 months, CMVneg n =28 , CMVpos n =46 .*

7.2.4 Gender specific differences

There were no gender specific effects on the proportions of T cells circulating *ex vivo* at any time point (data not shown). Both sexes responded similarly to SEB for all parameters studied, apart from an increase in IFNγ in boys at 9 months of age in the delayed vaccine group only ($p = 0.0199$) (Figure 7.11) demonstrating a non-specific pro-inflammatory sex difference according to vaccine schedule, however this was not significant after correction for multiple comparisons.

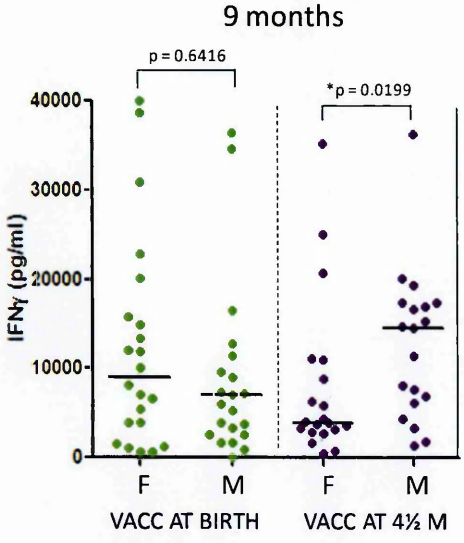


Figure 7.11: Gender specific SEB responses. *500 µL whole blood at 9 months of age was diluted 1: 5 and cultured for 5 days with SEB. At 9 months of age IFNγ responses were compared for gender differences in Group 1 (vaccinated at birth) and Group 2 (vaccinated at 4½ months). A Mann Whitney U test was applied to compare gender specific differences at 5% significance, the black bar represents the median value, *not significant after Bonferroni correction for multiple testing, Group 1 Female n = 22 Male n =21, Group 2 Female n = 20 Male n = 19.*

At birth, there were no gender specific differences in responses to any of the

mycobacterial antigens tested, apart from a trend for increased IFNγ production in response to PPD in boys compared to girls that was no longer significant after correction for multiple testing (p = 0.0355) (Figure 7.12).

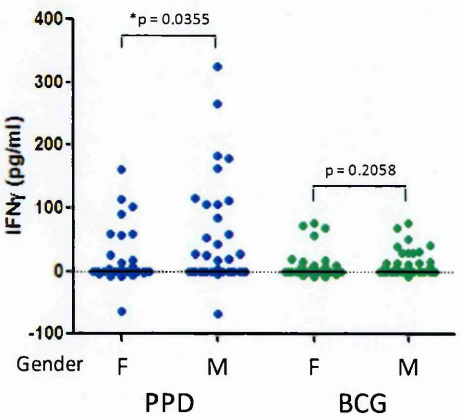


Figure 7.12: Mycobacterial specific gender differences at birth. *500 µL cord blood was diluted 1: 5 and cultured for 5 days with mycobacterial antigens PPD and BCG. IFNγ responses at birth were compared for gender differences (F = Female, M = Male). A Mann Whitney U test was applied to compare gender specific differences at 5% significance, the black bar represents the median value, *not significant after Bonferroni correction for multiple testing, Female n = 22, Male n =21.*

At 4½ months of age, BCG induced IFNγ was greater in males than in females in BCG vaccinated infants (p = 0.0038) with a similar trend but insignificant in response to PPD, p = 0.0767 (Figure 7.13A) suggesting boys have a greater Th1 response to BCG vaccination. Before correcting for multiple testing BCG induced Tregs (but not activated T cells) were also increased in Group 1 (vaccinated) in response to PPD and BCG in boys compared to girls (Figure 7.13B and C). In the unvaccinated group, males had increased levels of activated T cells and Tregs in response to *in vitro* BCG, although at low levels, but these values were not significant after adjustment for multiple comparisons (Figure 7.13B

and C). By 9 months of age there were no gender specific differences after adjustment for multiple testing in either group to BCG or PPD, however increased IFNγ (p = 0.0142) and a trend for increased IL-10 (p = 0.0346) were observed in Group 2 males in response to EC although the values were very low (Figure 7.13D).

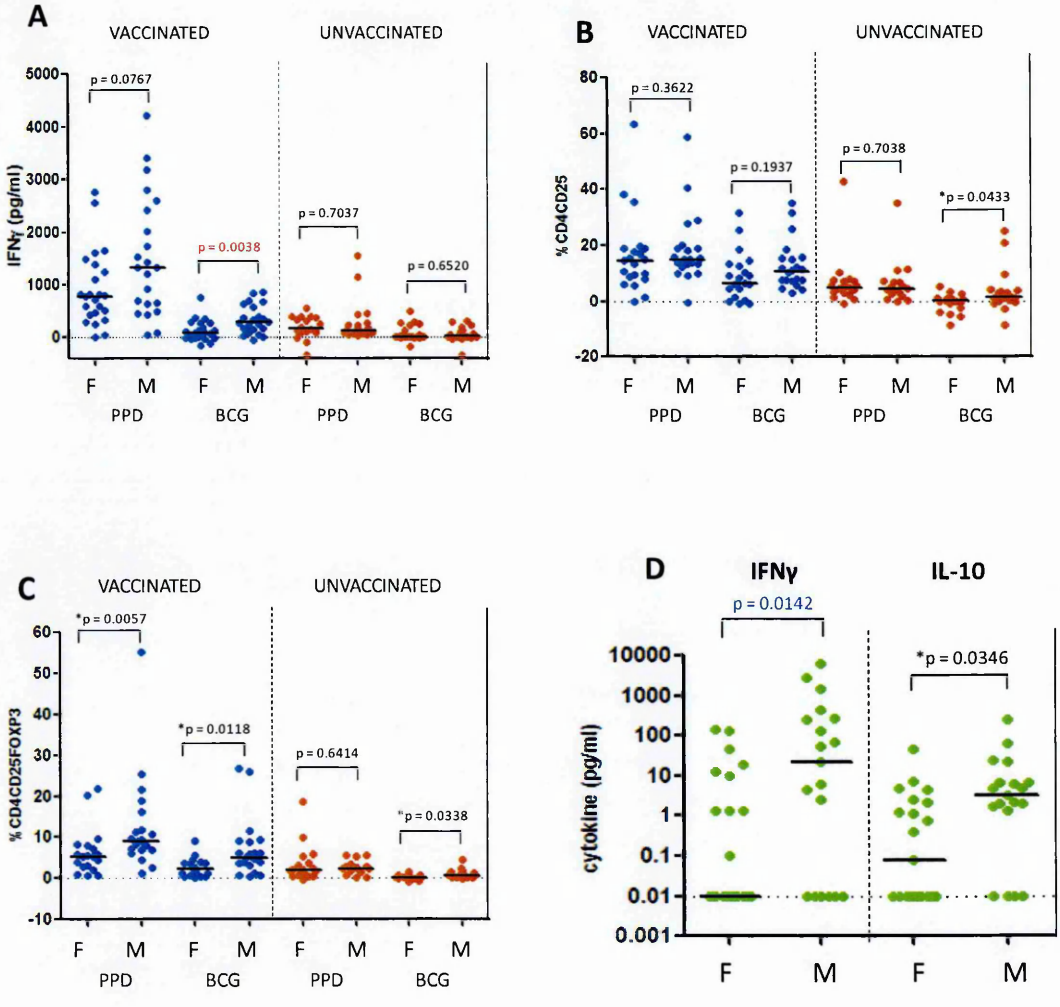


Figure 7.13: Gender differences at 4½ months of age. *500 µL whole blood at 4½ months of age was diluted 1: 5 and cultured for 5 days with mycobacterial antigens PPD and BCG. IFNγ (A), $CD4^+CD25^+$ (B) and $CD4^+CD25^+FOXP3^+$ (C) T cell responses were compared for gender differences (F = Female, M = Male) in BCG vaccinated and unvaccinated, Group 1(vaccinated) Female n = 19, Male n =22, Group 2 (unvaccinated) Female n = 18, Male n = 18. (D) IFNγ and IL-10 production from supernatants in response to ESAT-*

*6/CFP-10 fusion protein at 9 months of age in Group 2, Female n = 21, Male n = 19, (zero responses were given the value, 0.01 for illustration purposes). A Mann Whitney U test was applied to compared gender specific differences at 5% significance, the black bar represents the median value, *not significant after Bonferroni correction for multiple testing,*

Reactivity to the tuberculin skin test (TST) was not influenced by gender

There was no significant relationship between gender and reactivity to the TST in Group 1 at 4½ months of age (Fisher's Exact test p = 0.580) (Figure 7.14A). The presence of a BCG scar was also not related to gender. Only 5/50 (10%) individuals at 4½ months of age did not scar after BCG vaccination, 3 were female and 2 were male. At 20 months of age 7/85 (8.2%) children did not exhibit a scar, 3 were male and 4 were female (data not shown). However the median scar size for males was significantly larger (median 3 mm) than females (median 4 mm) (p = 0.0118) (Figure 7.14B) which supported a previous study in Malawi (Floyd, Ponnighaus et al. 2000).

A

		Female	Male
Group 1	**R**	**12**	**12**
Group 1	**NR**	**16**	**11**
Group 2	**NR**	**19**	**20**

NR – Non responders
R - Responders

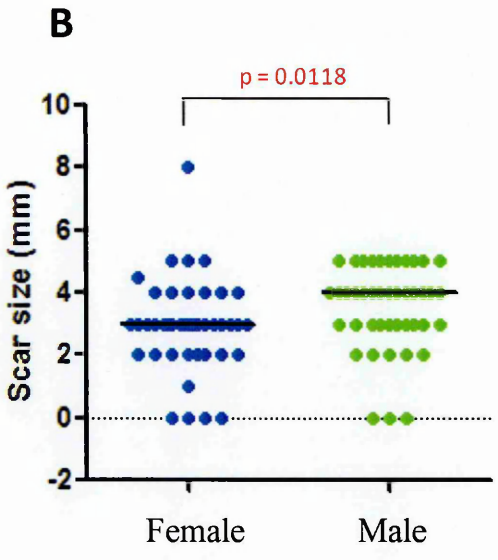


Figure 7.14: The relationship between Tuberculin Skin Test (TST), BCG scar formation and gender. *At 4½ months of age, 2 T.U. PPD-T was injected into forearm and induration was measured at 48 – 72 hours later, R = responders ≥ 5mm, NR = non responders < 5 mm. (A) numbers individuals in each group according to gender and response to TST. At 20 months of age a second TST was performed and scar size was measured according to gender (B). A Mann Whitney non-parametric test was applied at 5% significance.*

Gender did not relate to CMV infection

There was no gender specific difference associated with CMV infection at each time point (Fisher's exact test; at birth p = 0.3979, 4½ months, p = 0.0836, 9 months p = 0.4949). When separating into groups the same inference was observed at all time points (data not shown).

7.3 DISCUSSION

7.3.1 Age specific responses in unstimulated culture

Analysis for differences in the unstimulated day 5 culture across time points and according to vaccine group showed that at all ages responses were minimal, and often lower than observed prior to culture (*ex vivo*). It was evident that cord blood lymphocytes were highly proliferative and more susceptible to cell death, particularly the $CD4^+$ T cell population. This tendency for $CD4^+$ T cells to be more susceptible to apoptosis has been observed in studies of lymphocytic malignancies where $CD8^+$ T cells expanded better and but $CD4^+$ T cells had a high rate of apoptosis causing inverted CD4:CD8 ratios (Mazur, Davis et al. 2008). Previous studies have also observed spontaneous apoptosis in cord blood as a result of culture conditions (Tu, Cheung et al. 2000) (Yang, Hsu et al. 2001) (Canto, Rodriguez-Sanchez et al. 2003) which was greater than that observed in adult mice (Adkins, Chun et al. 1996; Canto, Rodriguez-Sanchez et al. 2003). The mechanism is thought to be IL-7 dependent and caspase-mediated (O'Neill, RM *et al*, 2003). Proliferation and increased apoptosis are also characteristics of recent thymic emigrants (RTE)(Hassan and Reen 2001). T cell receptor excision circles (TRECs) detected from the $CD4^+$ T cell population would help determine the contribution RTE make to the total T cell population.

The percentage of $CD8^+$ T cells in the lymphocyte population did not alter in cord blood so it is possible that these cells die at the same rate as $CD4^+$ T cells but are replenished more quickly in accordance with the higher proliferation rate observed among the $CD8^+$ T cell population in cord blood, and in support of the model described above (Mazur, Davis et al. 2008).

The total IL-10 production in the supernatant of unstimulated cells was not altered with age, however the proportion of IL-10 secreted from T cells was reduced suggesting that the source of IL-10 production in cord blood is not the same as in infants, and arises from a source other than T cells. Unstimulated day 5 IFNγ and IL-13 were produced at

lower levels than IL-10 at birth, but were significantly increased by 4½ months of age. This supports our mycobacterial stimulated results where IL-10 production was predominantly non-T cell derived.

A higher frequency of cord blood T cells produced TGFβ in unstimulated cultures suggesting a bias towards a more suppressive phenotype in culture. Increased *in vitro* TGFβ production observed in cord blood has been observed in others studies, particularly from $CD4^+$ T cells and has been associated with fish oil supplementation in pregnant women (Krauss-Etschmann, Hartl et al. 2008). Interestingly mothers in The Gambia have a diet relatively high in oily fish which may have contributed to this observation.

It was reassuring to note that the T cell changes that occurred during unstimulated cultures were not affected by the BCG vaccine schedule and could therefore be confidently used as background controls when analysing the mycobacterial stimulated responses in relation to BCG vaccination.

7.3.2 SEB responses in early life

SEB was found to stimulate T cells at all ages illustrating its ability to act as a strong global immune stimulus. CD4 expression on $CD3^+$ T cells was the only parameter that was consistently down regulated, an expected finding caused by the interaction between the SEB toxin and the TCR receptor leading to endocytosis of the TCR (Niedergang, Hemar et al. 1995). The only difference across age groups was that TGFβ was upregulated in infants but not in cord blood, although the background (unstimulated) levels were higher in cord blood compared to that in the infants as mentioned above. This confirms that SEB was an appropriate choice of antigen to be used as a positive control in our study. Although similar cells were involved in the immune response to SEB in the three age groups, by 9 months of age the magnitude of the response was greater than that observed in cord blood. SEB stimulates through the T cell receptor (TCR), suggesting that the lower response in cord blood was due to the immaturity of the immune cells and

dysfunction of interaction of the MHC/TCR complex. However there was an equivalent IFNγ response in cord blood in response to SEB compared to other time points which suggests that, irrespective of the immature immune system at birth, good Th1 responses are possible to specific stimuli. However it was not possible to identify the cellular source of the IFNγ and it is possible that innate NK cells may contribute to this production. The Th2 responses were weaker in cord blood. This supports studies by Chipeta *et al* that showed neonatal T cells were able to respond to TCR-mediated stimulation and to produce both Th1 (IFNγ) and Th2 (IL-4 and IL-13) cytokines compared to adults (Chipeta, Komada et al. 2000).

7.3.3 CMV infection

Nearly 100% of children are infected with CMV by 12 months of age yet they appear to be healthy and normal. The effects of such an early CMV infection are currently being investigated.

In this study the numbers of infants infected with CMV at 4½- and 9- months of age were comparable to previous reports in this population (Miles, van der Sande et al. 2007; Miles, Sanneh et al. 2008) however congenital infection was higher (previous study 4%, our study 16%). In our study neonates with congenital infection had enhanced IFNγ response to SEB as previously shown by Miles, *et al* at 9 and 13 months of age (Miles, Sanneh et al. 2008), although this did not correlate with an increase in activated T cells. This may indicate the effect is due to innate production of IFNγ, possibly from NK cells.

These previous studies in The Gambia also showed that CMV infection altered the differentiation of T cells, particularly $CD8^+$ T cells, and could influence responses to non-specific antigens (Miles, van der Sande et al. 2007; Miles, Sanneh et al. 2008). A greater number of $CD8^+$ T cells in the lymphocyte population of CMV infected infants prior to stimulation was observed in this study ex *vivo*, but less of them produced IL-10 in response to mycobacterial antigens compared to CMV negative infants. Mice models have shown

that low level IL-10 reactivity can lead to resistance to mycobacterial infection. Maybe the *ex vivo* increase in $CD8^+$ T cells and reduced IL-10 production in response to BCG at 4½ months of age after BCG vaccination provides the added advantage of increased resistance to *M.tb* infection. A study in Malawi suggested that CMV infection may explain why adolescents in Malawi (100% CMV infection) have lower naïve and higher memory T cells in circulating peripheral blood than adolescents in the UK (36% CMV infection) (Ben-Smith, Gorak-Stolinska et al. 2008).

7.3.4 Gender specific responses in early life

Associations found with gender suggested stronger immune responses (mostly IFNγ production) in males compared to females. Increased IFNγ production was observed at 9 months in the delayed vaccine group, suggesting a non-specific Th1 response 4½ months after delaying the BCG vaccine in boys compared with girls. IFNγ production and increased $FOXP3^+$Tregs *in vitro* were also found in males compared to females in response to mycobacterial antigens in Group 1 (vaccinated at birth) at 4½ months. Thus BCG appears to have some gender specific effect on both non-specific stimuli (SEB) and mycobacterial stimuli (BCG and PPD) whereby boys elicit a greater Th1 and $FOXP3^+$Treg response. Reactivity to the TST was not associated with gender which supports some of the literature (Floyd, Ponnighaus et al. 2000; Santiago, Lawson et al. 2003; Okan, Karagoz et al. 2006). In a TB case contact study in The Gambia the probability of TST reactivity was higher in males and increased with age however this study was not restricted to young infants (Lienhardt, Fielding et al. 2003). BCG scar size has also shown gender bias with larger scars in males than females (Floyd, Ponnighaus et al. 2000), and indeed in our study males had larger scars than females. Non-specific beneficial effects of BCG vaccination on morbidity and mortality have shown a greater benefit in girls. One explanation for this may be that girls have a better ability to control overt inflammation.

7.3.5 Effects of seasonality on immune responses in early life

Recent data has suggested that immune responses may be affected by seasonality. Lalor found that both in the UK and in Malawi, infants born during hotter and drier times of the year were more likely to produce a positive IFNγ response (> 62 pg/mL) to PPD 3 months after BCG vaccination (Lalor, Ben-Smith et al. 2009). In a separate study, Miles *et al* found that higher proportions of $CD4^+$ T cells expressing CD154 (CD40L, responsible for killing of monocytes and macrophages after mycobacterial infection) at 12 months of age was higher in those children born within the wet season compared to the dry season (Miles, van der Sande et al. 2008), however the variation within individuals was very high in this study. This study took place within the same community of The Gambia as our study and therefore using the same definitions of seasons, wet (June to October) and dry (November to May), our study showed roughly equal numbers of children born in each season (wet n = 59 and dry n = 44) and no correlation between season and IFNγ responses to PPD 4½ months after BCG vaccination (data not shown). However at the same age, BCG naïve infants showed a significant increase in IFNγ responses to PPD if born in the wet season compared to the dry season ($p = 0.0146$, dry n = 20, wet n = 18). The wet season is also very humid and is often associated with increased microbe survival which may indicate the NTM exposure is higher in the wet season. A larger sample size is necessary to examine this theory in more detail.

In summary, cord blood T cells are highly proliferative but are more susceptible to cell death than at subsequent time points. Cord blood T cells are immature but can still elicit responses to SEB of a similar magnitude to infant responses. Gender specific responses showed a BCG related trend towards males having a greater IFNγ response to non-specific and mycobacterial specific stimuli.

CHAPTER 8

Discussion

8.1 OVERALL DISCUSSION

BCG vaccine is administered at birth in places that TB is endemic. Although BCG is the oldest vaccine and has the widest coverage, TB has continued to be on the increase underlining its inadequacy to protect against the disease. Immaturity of the immune system of the newborn recipients of BCG and interference of environmental NTM have been the major reasons suggested for the inability of BCG to protect against TB throughout life, particularly in places where TB is endemic. This study used BCG vaccination to further elucidate some of these hypotheses, as well as using it as an immunogen in early infancy to further characterise the magnitude and quality of immune responses of newborns and factors that could influence them. This study is one of the few longitudinal studies assessing BCG immunogenicity at different time points in the first 9 months of life. The strength of this study includes the large number of subjects that were successfully followed up, and the detailed characterisation of the T cell responses.

8.1.1 NTM effect on BCG immunogenicity

Delaying the BCG vaccine to 4½ months of age compared to vaccination at birth made it possible to examine the contribution of background mycobacterial responses on BCG immunogenicity. *In vitro* PPD reactivity was used as a surrogate for NTM exposure since it is a mycobacterial protein containing many antigens present in NTM. Prior to BCG vaccination the delayed vaccine group had a significant upregulation of $CD4^+CD25^+$ and $CD4^+CD25^+FOXP3^+$ T cells, and increased production of IFNγ and IL-13 in response to PPD, accompanied by a borderline increase in IL-10 production, suggesting that immune priming by NTM in the first 4½ months of life leads to pro-inflammatory and regulatory T cell responses. All these children were anergic to the TST, suggesting that NTM exposure by this age was not able to prime a TST response. Thus, although PPD memory T cells existed in the peripheral blood of these children the mechanisms required to home to the skin may be impaired. Skin allergy studies have shown that Tregs expressing homing

markers such as CLA home to the skin (Colantonio, Iellem et al. 2002; Cavani, Nasorri et al. 2003), and perhaps this mechanism is responsible for the TST anergy.

IFNγ responses to mycobacterial antigens, assessed 4½ months after BCG vaccination, were reduced in the delayed vaccine group compared to the group vaccinated at birth. This finding supports our original hypothesis that exposure to NTM prior to BCG vaccination attenuates BCG immunogenicity. It is possible that IL-10 is responsible for the suppressed IFNγ response observed after BCG vaccination in this group (measured at 9 months of age), possibly by the induction of adaptive (IL-10 producing) Tregs rather than naturally occurring $FOXP3^+$ Tregs. Black *et al* previously found in Malawi, higher IFNγ responses to PPDs from the MAIS NTM complex prior to BCG vaccination related to lower IFNγ responses to PPD-T 1 year after immunisation (Black, Dockrell et al. 2001). In our study, responses to PPD at 9 months of age did not reveal any correlation to their PPD responses prior to BCG vaccination. This included regulatory responses ($CD4^+CD25^+FOXP3^+$ or IL-10 production) that were hypothesised to be involved in attenuation of BCG immunogenicity. When comparing geographical locations with different BCG protection rates it was suggested that the immunogenicity of BCG is related to the difference between PPD responses pre- and post- BCG vaccination, rather than the cross sectional response in isolation (Black, Weir et al. 2002; Weir, Black et al. 2006). Our study supported this by comparing the responses to PPD pre- and post- BCG vaccination in each group. This difference was less in the delayed vaccine group compared to BCG vaccination at birth suggesting the delayed vaccine group may be less protected. The strains of local NTM appear to be important in their effect on BCG responses in that those closely related are more likely to elicit cross-reactive immunity. Interestingly the most common strains of NTM in The Gambia are *M. intracellulare* and *M. avium* (Corrah 1994), and animal studies have shown that exposure to *M. avium* can limit BCG growth and reduce protection levels (Brandt, Feino Cunha et al. 2002; Buddle, Wards et al. 2002; de Lisle, Wards et al. 2005).

8.1.2 In utero exposure to NTM

It is unknown if mycobacterial antigens can cross the placental barrier or are transferred to the infant through the breast milk, but there are several studies that elude to this being the case as discussed in Chapter 6. If the foetus is exposed to NTM *in utero* it is plausible that responses might even effect BCG vaccination at birth as well as at 4½ months of age. There are no published reports of a comparison of cord blood mycobacterial reactivity between high NTM and low NTM geographical settings, but preliminary data show slightly higher PPD responses in the cord blood of Malawi neonates compared to UK neonates (Hazel Dockrell, personal communication) although still low suggesting that *in utero* exposure might play a role and supports the PPD responses that were observed in our results.

8.1.3 Role of cytokines in BCG immunogenicity

The Th1 cytokine, IFNγ is thought to be key in conferring protective mycobacterial responses. The opportunity to study cytokine responses in BCG vaccinated and naïve children was possible at 4½ months of age. At this age several cytokines were examined after mycobacterial stimulation including IFNγ, IL-10, IL-13, IL-6, IL-17 and IL-7. The results indicate, as many recent studies have also shown, that although IFNγ is important, it is possibly not sufficient to understand the mechanisms of BCG immunity and protection. It was confirmed that BCG vaccination in early life can induce a Th1 response at 4½ months of age but the results show that the sustained memory response to BCG vaccination is Th2 skewed, as often observed in early life to immune stimuli. It was interesting that the Th2 cytokine, IL-13 was induced by BCG vaccination when administered at either time point and, in contrast to IFNγ; reactivity was sustained to 9 months of age in those vaccinated at birth. Other studies found similar IFNγ results when delaying BCG to 2 or 4½ months of age although, in contrast to our results, the Th2 (IL-4,

IL-5 and IL-13) responses were low(Marchant, Goetghebuer et al. 1999; Ota, Vekemans et al. 2002). IL-4 is often measured as a Th2 cytokine, but IL-4 protein and mRNA levels in peripheral blood are usually low (in house communication). IL-13, another Th2 cytokine, utilises the same IL-4Rα receptor suggesting that their function is related. Rook *et al* propose that BCG fails in people that have high pre-existing levels of IL-4 because it cannot downregulate the subversive Th2 component, and can only boost the Th1 response which may not be sufficient for protection (Rook 2007). In support of this theory, therapeutic vaccine trials in mice that suppress the Th2 response have shown promise whereas BCG was therapeutically inactive (Lowrie, Tascon et al. 1999; Hernandez-Pando, Jeyanathan et al. 2000). It is possible that the ratio of Th1 to Th2 responses is more important than absolute concentrations. Increased IFNγ: IL-5 ratios after BCG vaccination correlated with cytotoxic activity, suggesting that it may be associated with the protective activity of BCG (Hussey, Watkins et al. 2002).

Recently IL-17 production from T cells (Th17) has been shown to be involved in the immune response to mycobacteria, although studies are conflicting as to its role in protection against TB. Human TB patients have reduced levels of IL-17 (Scriba, Kalsdorf et al. 2008; Sutherland, Adetifa et al. 2009), but mouse studies have shown increased IL-17 levels in IFNγ knockout mice that do not confer protection (Cruz, Khader et al. 2006). However IL-17 together with IFNγ may be important in the secondary response to TB, as suggested in mice that were protected by a peptide based TB vaccine (Khader, Bell et al. 2007). Our results showed an approximate 10-fold increase in IL-17 production in the BCG vaccinated group at 4½ months of age, but the levels were low (BCG vaccinated median value 14.55, unvaccinated median 1.53). It is not clear what concentration of IL-17 is needed to confer protection, but it is feasible that such levels are adequate. We did not study IL-17 levels at 9 months of age, but it would be interesting to study the effect of delaying the BCG vaccine on IL-17 production.

At 4½ months IL-6 was induced in response to PPD in both groups; BCG vaccinated group by138-fold and unvaccinated group by 10-fold. IL-6 production suggests a role for innate responses in BCG immunogenicity, as may be expected. IL-6 is dependent on stimulation via TLR2 and TLR4 which are common receptors that recognise bacteria, including BCG (Jang, Uematsu et al. 2004). It can play a role in T cell activation and suppression of regulatory T cells (Pasare and Medzhitov 2003). However production of IL-6 from *M.tb* infected macrophages from mice can inhibit the IFNγ responsiveness of neighbouring macrophages by selective inhibition of a subset of IFNγ responsive genes (Nagabhushanam, Solache et al. 2003). In this study IFNγ itself was unaffected, but IL-6 was described to have Th2 polarising effects (Jones 2005). These results are of direct relevance to the results of our study in which both IFNγ and IL-6 were upregulated after BCG vaccination. In humans increased levels of IL-6 have been found in the plasma of TB patients, suggesting that induction of IL-6 is another pathway that *M.tb* uses to evade the immune response. In the case of BCG vaccination IL-6 induction may be linked to reduced protective responses which would not be evident when examining IFNγ responses alone. In this context it should be noted that the birth process can initiate an acute phase reaction in the newborn child including upregulation of IL-6 (Marchini, Berggren et al. 2000). Furthermore, the human newborn has a higher IL-6/TNFα ratio compared to adults (Angelone, Wessels et al. 2006). It is possible that these high levels of IL-6 initiated at birth contribute to that induced by BCG vaccination.

IL-10 has a major immunomodulatory role in that the absence of IL-10 is associated with immunopathology and autoimmune diseases, while the enhancement of IL-10 reactivity can inhibit the immune response to pathogens (reviewed in (O'Garra, Vieira et al. 2004)). The effects of IL-10 in regulating infection are often due to reduction in TNFα which reduces several secondary inflammatory mediators such as chemokines and prostaglandins, and enhances anti-inflammatory molecules. IL-10 can also inhibit antigen presentation and downregulation of TLR4 (as reviewed in (Moore, de Waal Malefyt et al.

2001)). In addition the reciprocal relationship between IFNγ and IL-10 seems critical in the clearance of *M.tb.* This was shown in a prospective case contact study in Pakistan where contacts that developed disease 12 months after exposure had lower IFNγ/ IL-10 ratios compared to contacts that did not develop disease (Hussain, Talat et al. 2007). The IL-10 may act via inhibition of antigen presenting cell (APC) activation, therefore reducing the Th1 pro-inflammatory effects however direct inhibitory effects on T cells have also been demonstrated (O'Garra, Vieira et al. 2004). Our results indicate that it may have a prominent role in controlling the strength of the immune response to vaccination and in particular, help regulate tuberculin skin test induration. However it is also evident that IL-10 can be produced from many different cell types including T cells, ($CD4^+$ and $CD8^+$, Th2, Tr1, Th1, Th17), macrophages, dendritic cells, B cells, some granulocytes and non-immune cells (keratinocytes, epithelial cells and tumour cells) making it difficult to understand the specific role IL-10 plays in controlling infection. The source of IL-10 may also differ according to age. In our study it was found that $CD8^+$ T cells produce more IL-10 than $CD4^+$ T cells at 9 months of age in response to SEB but the reverse was true in cord blood. The IL-10 producing T cells were not assessed for suppressive activity but it is possible that the $CD4^+$ IL-10 producers represent Tr1 cells. The $CD8^+$ IL-10 producing T cells could relate to the original suppressor cells observed in the 1980s (Jiang and Chess 2004).

It was interesting to find that in cord blood, basal IL-10 production (from the supernatants of unstimulated cultures) was greater than both IFNγ and IL-13, but remained similar when compared to the later time points. This is in contrast to basal levels of IFNγ and IL-13 that were both increased significantly at 4½- and 9- months of age compared to at birth suggesting IL-10 production at birth is robust. Interestingly lymphocyte production of IL-10 is low at birth, particularly from $CD8^+$ T cells, suggesting that the source of IL-10 is non-T cell derived at this time. This agrees with data from South African neonates where IL-10 production in cord blood in response to BCG was produced predominantly by

monocytes and the IFNγ was produced by NK cells (Watkins, Semple et al. 2008). It is possible that differences in the cytokine milieu influence the polarisation of the T cell responses at different ages. IL-10 was induced in the supernatants of EC stimulated cultures in unvaccinated but not vaccinated children at 4½ months. In fact down regulation of IL-10 was only seen in the BCG vaccinated group suggesting BCG vaccination may override the IL-10 regulatory effects to mycobacterial exposure.

Our lack of BCG-induced IL-10 did conflict with many other BCG vaccination studies in both animals and humans that found BCG induced a strong IL-10 response (Gagliardi, Teloni et al. 2005; Sendide, Deghmane et al. 2005; Soares, Scriba et al. 2008). However a lack of unvaccinated controls in many human studies means it is not possible to attribute all IL-10 induction to BCG vaccination where some may be due to NTM exposure prior to vaccination (Soares, Scriba et al. 2008). If NTM induces IL-10, this could suppress antigen presentation and TLR4 and thus reduce the response to subsequent BCG vaccination.

Since the correlates of BCG protection are still unknown, we cannot say whether the cytokine findings in this study are related to protection, but it is becoming more evident that IFNγ may not be the only cytokine responsible for protection. With the advancement of new technologies, a cytokine profile or signature consisting of a combination of cytokines is more likely to be used as a biomarker to predict protection and susceptibility to *M.tb*.

8.1.4 Tuberculin skin test (TST)

In the vaccinated group, 50% of the children did not respond to the TST at 4½ months of age. These TST anergic children produced less IFNγ in response to PPD and showed a trend for higher levels of IL-10 compared to the TST responders. It is possible that these children had a stronger response earlier and that it had already waned by 4½

months of age. Adult studies have shown that IFNγ responses peak at 4 weeks post BCG vaccination and decline by 12 weeks (Nabeshima, Murata et al. 2005). Interestingly IL-10 inversely correlated with TST induration, whereas IFNγ bore no correlation. We propose that IFNγ initiates the skin test, but IL-10 controls the induration possibly via production from macrophages that home to the site of challenge. Our study used 10 mm as a 'cut off' as indicating potential *M.tb* infection, however 50% of TST responders were ≥10 mm at 4½ months of age and only 2 of these children were suspected as being exposed to TB. We therefore suggest that a higher 'cut off' is required in early life for BCG vaccinated children when being used as a diagnostic indicator of potential *M.tb* infection. This is in agreement with the study by Chan *et al* that suggested using TST 'cut offs' for ages 0 – 1, 2 – 3, 4 – 5 and 6 – 7 years of respectively 21, 18, 13, and 10 mm respectively (Chan, Chang et al. 2008). Use of these values in our study would suggest that no one was infected with *M.tb,* which is the likely scenario. A 'cut off' for the skin test at 20 months of age, of 18 mm would, also indicate that only one subject was *M.tb* infected, which in fact was later confirmed to be the case.

It is possible that peaks of TST induration predict the type of exposure where 5 – 9 mm indicates exposure to NTM, 10 – 12 mm predicts BCG vaccination and > 13 mm indicates exposure to *M.tb*, however this would be determined by the age of the subject and the timing of the BCG vaccination.

8.1.5 Non-specific effects of BCG

The changes in the timing of BCG vaccination in our study had no effect on cross sectional *ex vivo*, unstimulated and SEB stimulated cell populations at each time point. This agrees with others who analysed circulating immune cell subsets in infants pre- and 7 months post- infant vaccinations (Berrington, Barge et al. 2005).

It was not clear from our results if there are gender differences in BCG immunogenicity. There was a trend for males to have greater pro-inflammatory responses to BCG, but this finding was only statistically significant at 4½ months of age in the BCG vaccinated group in response to the BCG vaccine *in vitro*.

8.2 LIMITATIONS OF THE STUDY

The project was designed as a prospective observational study assessing BCG immunogenicity in the context of pre-sensitisation from NTM. Although the results have shown many interesting concepts, there were certain financial, technical, time, and personnel limitations that are addressed below.

Blood collection

As with most infant studies a restriction on blood collection of 1 mL/ kg weight is generally thought ethically acceptable which meant that only 5 mL (or less in some cases) of venous blood was collected from the children, thus limiting the numbers of experiments that could be performed. The timing of the follow up bleeds was determined by what was considered an acceptable frequency that a child should be bled. This was discussed with the community fieldworkers and the ethics committee and chosen accordingly. It is possible that measuring responses prior to 4½ months of age would have provided more or different information about immune responses to BCG in early life.

Treg definition

The assays were designed when the only available flow cytometer was the FACSCalibur which detects only four fluorochromes thus limiting the number of cell markers that could be measured. This limited our definition of Tregs according to surface and intracellular markers. At the commencement of the study the most appropriate Treg definition was $CD4^{+}CD25^{+}FOXP3^{+}$. Recently the addition of low CD127 expression has been proposed as a more precise phenotype for Tregs. It was not possible to measure certain antibodies in the same assay due to differences in staining protocols; for instance the anti-FOXP3 antibody protocol from e-Bioscience had a tendency to increase background staining of other intracellular markers (including, Ki-67, IL-10 and TGFβ) if used in the same assay.

Although FOXP3 has been used as a marker of Tregs, it is still not apparent if all FOXP3+ T cells are functionally suppressive. Assays involving the separation of T cell subsets and culturing with and without Tregs require large volumes of blood, and was not possible in our study. Furthermore it was not possible to purify $FOXP3^+$ cells without a phenotypic cell sorter which is not available in The Gambia. The use of Ki-67 as a marker of proliferation was assessed but it was not possible to add this antibody to the FOXP3 panel for the reasons described above. The limitation of this marker is that it only indicates cell cycle division at the end of the culture period.

With the recent purchase of the CyAn (Dako, CA, US) it is now possible to stain for 9 different markers within each panel. This would have allowed us to identify other populations of cells that might be involved in BCG immunogenicity, such as macrophages, NK cells and dendritic cells.

Efficacy of BCG vaccination

As with all human studies in the field of TB, without the identification of correlates of protection, observed immune responses can only indicate how the immune system is reacting to a stimulus. Although we found differences in responses to the BCG vaccine administered at different times, the relationship to protection against TB could not be determined.

RNA expression profiles

The study design described in the Materials and Methods mentioned analysis of RNA expression profiles from 100 µL of the 5 day cultures. Extraction of the RNA from about 50% of these cultured samples yielded low concentrations and in some cases RNA of poor quality as assessed by the Agilent 2100 Bioanalyser (Agilent, Santa Clara, CA, US) by our collaborator, Dr Roger Buxton at National Institute for Medical Research, London,

UK. It was thought that this may be due to the small quantity of samples or the presence of haemoglobin in the samples degrading the RNA during the collection and/or thawing of the samples. For this reason, microarray or real time PCR analysis was not performed on these samples and therefore not presented in this thesis.

Genetic heterogeneity

The heterogeneity of the responses illustrates that although there may be an overall pattern of reactivity observed, not all individuals behave in a similar way (illustrated in Chapter 5, Table 5.4). IL-10 responses were particularly heterogeneous in our study. Natural variation in The Gambian population has been observed in responses to several mycobacterial antigens 2 months of age after BCG vaccination at birth (Finan, Ota et al. 2008). This becomes increasingly important when analysing responses using small numbers of subjects, as the statistics may miss important findings. Furthermore, those individuals who behave differently may be of particular interest.

Time constraints

Finally, one big limitation of this type of work is the time constraints of performing very labour intensive immunological assays on fresh samples every day. As a result the number of samples collected each day was limited and sample collection took 24 months to complete.

8.3 FUTURE STUDIES

This study has generated a number of new ideas that warrant further study. Furthermore, a number of the limitations discussed above could be overcome in future studies due to newer technology and additional published literature in this area. Despite this few immunological studies have examined the development of the immune system in early life and therefore this is an exciting field with much more work to be done.

8.3.1 Effect of BCG on responses to other EPI vaccines

Plasma samples were collected at each time point from the study participants. Antibody responses to other vaccines delivered as part of the EPI schedule could provide data on the effect delaying BCG has on responses to these vaccines. BCG has previously been shown to increase the antibody response to hepatitis B vaccine (HBV) and oral polio vaccine (OPV), dependent on the timing of the vaccines; delaying the BCG vaccine to 2 months of age reduced the HBV antibody enhancement but increased the OPV antibody production (Ota, Vekemans et al. 2002). In addition non-specific effects of BCG administered at the same time or prior to OPV were observed in Guinea Bissau (Aaby *et al*, unpublished). Financial and logistical constraints have delayed this work but it is expected that it will be completed within the next year.

8.3.2 Role of IL-10 in mycobacterial immunity

The contribution of IL-10 to immune responses in early life is not clear but our studies suggest it may play an important role in the control of pro-inflammatory responses. It is therefore important to establish what cells are involved in producing the observed IL-10 at the different time points. Access to 9 colour staining would allow us to assess for ICS IL-10 production among monocytes, macrophages, dendritic cells and T cells in one sample. Functional assays could also be carried out using neutralising anti-IL-10 antibodies

and examining *in vitro* effects to understand the underlying mechanisms by which IL-10 exhibits its immunoregulatory role.

8.3.3 Novel CD4CD8 double positive T cell population

While analysing the data two populations of CD4CD8 double positive (DP) T cells were evident both *ex vivo* and after 5 days of culture. *Ex vivo* the DP population was mainly $CD4^{hi}CD8^{int}$ and consisted of approximately 1 – 2% of lymphocytes (Figure 8.1A), whereas after 5 days of SEB stimulated culture it was predominantly $CD4^{int}CD8^{hi}$ (Figure 8.1B) among approximately 2 - 4% of the lymphocyte population. This DP population appeared to be highly proliferative after stimulation with SEB (27 – 37% of lymphocytes at all ages) and a considerable proportion of these cells expressed FOXP3 (median 18 - 25% at all ages) and IL-10 (median -7 - 3.75%). This population is rare in human peripheral blood due to the efficient selection process in the thymus whereby the DP population loses either CD4 or CD8 expression before entering the periphery (Blue, Daley et al. 1985; Blue, Daley et al. 1986; Patel, Wacholtz et al. 1989; Kay, Bone et al. 1990; Sala, Tonutti et al. 1993). However this population is abundant in pigs (in the adult pig 60% of T cells are DP) where they mainly express IL-10 (Hernandez, Garfias et al. 2001). These DP cells have been found in individuals with chronic viral infections (Macchi, Graziani et al. 1993) including HIV (Lusso, De Maria et al. 1991) and malignancies (Mizuki, Tagawa et al. 1998). An extrathymically-derived DP γδTCR cell population has also been found to be associated with intestinal epithelium (reviewed in (Kagnoff 1998)). The proportion of γδTCR cells that are DP in humans is not known. A recent study has shown T cell clones developed from cord blood in an IL-2-independent manner and co-express CD25 and FOXP3, suggesting that they are another subset of regulatory T cells (Nakamura, Suzuki et al. 2007). DP Tregs have been postulated in the MHC class II KO mouse, whereby $CD4^{+}CD8\alpha\beta^{+}CD25^{+}$ αβ T cells attenuate $CD4^{+}$ T cell-induced transfer colitis (Krajina, Leithauser et al. 2004). Further studies to characterise this population in our setting are

proposed, including identifying if these are more common in young children and related to recent thymic emigrants (RTE).

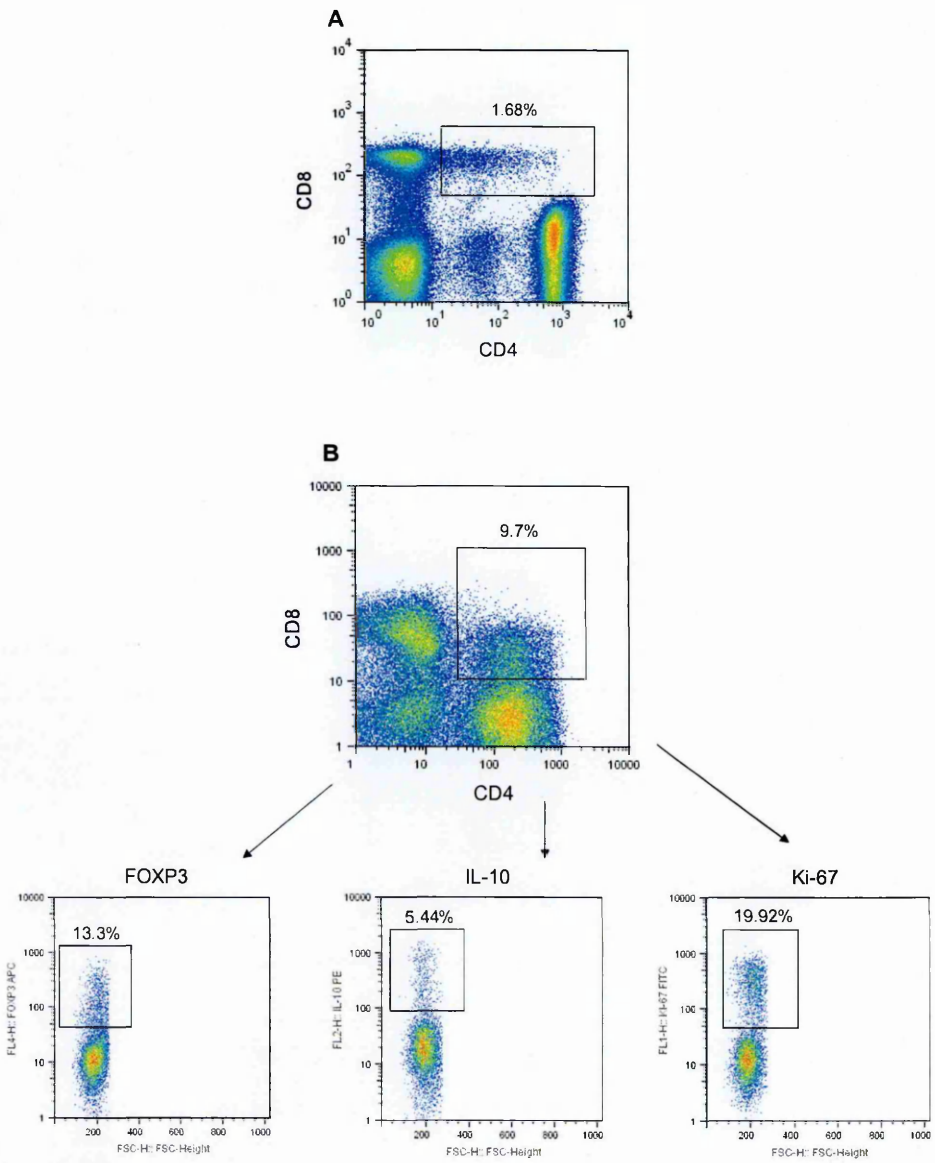


Figure 8.1: A representative plot illustrating the CD4CD8 DP T cell populations. *The proportion of lymphocytes that possess the phenotype $CD4^{int}CD8^{hi}$ ex vivo (A) and $CD4^{hi}CD8^{int}$ and after 5 days of culture with SEB (B) showing the percentage of these DP cells that express FOXP3 (left), IL-10 (middle) and Ki-67 (left).*

8.3.4 Innate immunity in infants

Modifying the innate response may increase the strength of the longer lasting, more specific adaptive memory response and vaccination is no exception to this. Microbial products activate the host cells via TLR present on cells of the gut, respiratory system and in blood. When triggered by the microbial product, TLR activation initiates cell maturation, translocation of NF-kB, transcription of multiple genes and production of cytokines including IFNγ and TNFα which are associated with a strong Th1 inflammatory response (Barton and Medzhitov 2003). Indeed a number of studies show that polymorphisms in human TLRs and their adaptor molecules cause increased susceptibility to a broad range of bacterial, viral, fungal and protozoan pathogens (Misch and Hawn 2008).

Despite normal basal expression of TLR and associated signalling intermediates, neonatal responses to a range of TLR agonists are characterised by impaired production of pro-inflammatory cytokines, such as TNFα (Levy, Zarember et al. 2004; Angelone, Wessels et al. 2006). Studies of newborns based in the US have shown very poor reactivity to all TLR agonists except for TLR8, raising the possibility that TLR8 might be suitable for development as a vaccine adjuvant in this age group (Levy, Zarember et al. 2004; Levy, Suter et al. 2006). This is thought to be due to the distinct neonatal adenosine system, including relatively high adenosine concentrations in neonatal blood plasma which also leads to preserved IL-6 production (Levy, Coughlin et al. 2006). However there are no such studies in African children where improved vaccine schedules are urgently required, and who may also give a different response profile to a Western population.

In collaboration with Dr Ofer Levy, Harvard Medical School we proposed to carry out a cross-sectional prospective study that will examine cytokine immune responses to a panel of TLR agonists in different age groups; cord blood, 1-, 2-, 3-, 4-, 6-, 9- and 12-months of age, using the CBA multiplex system. We hypothesise that while the responses

to TLR agonists are limited at birth, increased reactivity to a broader range of agonists will occur within the first few months of life in this setting of high exposure to pathogens. This work will provide vital information for those developing TLR agonists as adjuvants for infant vaccines.

8.4 CONCLUDING REMARKS

The final conclusions of this study support the original hypothesis that delaying BCG vaccination to 4½ months of age reduced the post-vaccination pro-inflammatory Th1 response, which may in turn cause reduced immunogenicity of the vaccine. It was hypothesised that Tregs are responsible for the attenuated response. Our results suggest that while $FOXP3^+$ naturally occurring Tregs may not be involved, IL-10-producing Tregs induced in response to NTM exposure may be responsible for the reduced IFNγ response after vaccination. It was not determined if this was from Tr1 regulatory T cells, or other IL-10 producing T cell subsets.

BCG is administered at birth in countries with a high risk of TB, however there is no immunological evidence to suggest that this provides better protection against TB compared to immunising later in infancy. Our study could not determine the effect of delaying the BCG vaccination on protection against TB, but does provide immunological evidence that supports vaccinating at birth, since it induced a stronger Th1 IFNγ production 4½ months post vaccination compared to delaying the vaccine. However responses by 9 months of age were comparable in the two vaccine groups suggesting that long term protection may not be affected by delaying the vaccine.

This study therefore provides valuable insights into BCG immunogenicity in early life and the effect of delaying the vaccine. This is the most comprehensive analysis to date of how this relates to regulatory T cell activity and interestingly to Th17 responses. It has provided new information into the mechanisms behind the control of TST reactivity, and

concurs with the suggestion of others that 'cut off' values need to be higher in these young age groups. It has opened up a number of questions and future avenues of research. In particular, the role of CD4CD8 DP regulatory T cells in early life, and the nature of the IL-10 producing Tregs that seems to play a regulatory role in mycobacterial immunity in this age group. This clearly demonstrates the importance of studying the immune system in this young age group in whom the majority of vaccines are administered.

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APPENDIX I: Study Information Sheet for BCG Study

Infant BCG study SCC1009

Study information sheet

We would like to start by thanking you for helping us with the study that you are already enrolled on. However, we would like to ask you to accept a few changes in the sampling schedule that will help us to learn about one of the vaccines your baby receives called BCG.

We would like you to help us better our understanding of BCG vaccination and prevention of TB (tuberculosis). We would like to inform you about the study and how you can help us.

What are vaccinations?
Most children in The Gambia, including your child, receive vaccinations through the EPI programme. These vaccines protect them from diseases that may be dangerous when they are young but also later in life. Sometimes our body cannot control an infection on its own and that is why vaccinations are administered. Vaccinations work by teaching cells to recognise and fight an infection the next time you come into contact with it.

Why are we interested in BCG vaccination? BCG vaccine is the only vaccine against TB but it isn't very effective in African countries therefore better vaccines are required. TB is a disease caused by bacteria that infects the lungs. TB is transmitted from person to person through breathing the air that is infected with these bacteria. One-person in every ten infected people progress to disease during their lifetime, leading to two million deaths a year worldwide. In the Gambia, BCG vaccination is given at birth and provides some protection against developing serious forms of TB, although in adults the protection may be very low. The reason for this is unknown but we think it may relate to how specific cells develop in the infant at an early age in The Gambia, and this is what we would like to investigate.

What is the aim of the study?
Our aim in this study is to understand the regulation of the immune responses to BCG early in life so that we can we can help develop a vaccine that will be able to protect children and adults in The Gambia.

Guidelines for BCG vaccination suggest it should be given within the first year of life to protect against childhood TB and therefore we would like to look at two different times of vaccination to see what effect vaccinating at different times has on immunity to TB. Your baby will either be part of group 1 and be vaccinated at birth, or part of group 2 and vaccinated at 4.5 months. This will enable us to study the development and regulation of TB immunity before or after BCG vaccination.

What happens if you agree to participate? Your participation in this study is entirely voluntary. We will continue to provide vaccines for your child according to the EPI programme and your child will receive free healthcare throughout the duration of the study.

We would like to collect 50ml of cord blood at birth and then we would like you to bring your baby back to the clinic twice more at 4.5-, and 9- months of age to collect a maximum of one teaspoon (5ml) of blood from them. At 4.5 months we would also like to perform a

standard skin test on your child for their reaction to the vaccination. At all time points we would like to collect a urine sample to test for current CMV infection (a common infection that may influence your response to vaccines). If you have been told that your child is part of group 1 then they will be vaccinated with BCG at birth, and if they are part of group 2 then they will be vaccinated at 4.5 months.
We will monitor your child on monthly visits as part of a routine health check and also for TB exposure.

Problems
If there is any reason to suspect your child has been exposed to TB then we will assess your child thoroughly before allowing your child to continue in the study.

BCG vaccination has been widely used for many years and therefore we do not expect any problems with BCG vaccination at either time points. One teaspoon of blood will be replaced in the body within a few hours and therefore the child will not be affected by collecting 1 teaspoon of blood. We guarantee this blood will not be used for anything other than this study without consent from you.

MRC Contacts. If you are unable to attend the clinic on the designated times then please call the Sukuta Health clinic or Katie Flanagan or Sarah Burl on the telephone numbers below and inform the person on duty. We will try and arrange for blood to be collected at an alternative time.
Please feel free to ask any questions you like at any time during the study. You may ask the field worker that visits you, or the MRC doctor in the health centre. You can also phone Dr Katie Flanagan or Ms Sarah Burl at MRC (4495442 ext.314 or 379 respectively).

Your rights. All information that you give us will be treated as confidential. You are free to leave the study at any time you wish, which will not affect your access to normal medical care. We will compensate your travel costs to the clinic during this study. **If your child is part of group 2 that is scheduled to be BCG vaccinated at 4.5 months it is imperative that you bring your child back for BCG vaccination even if you do not wish to participate in the study any longer. It is also VERY IMPORTANT that you inform us of any travel plans before the 4.5 months to help us ensure your child is vaccinated with BCG.**

This study (SCC1009) is funded by MRC and has been approved by the MRC and LSHTM Ethics Committees.

If you are willing to help us with this study, please sign the consent form.

Thank you very much.

APPENDIX II: Consent Form for BCG Study

Infant BCG study SCC1009

CONSENT FORM

I have read the information sheet fully, and/or it has been read and explained to me and I have had a chance to ask questions about the study and had them answered.

I willingly and voluntarily agree to allow my child to participate in this study.

I understand my child is free to leave the study at any time, and that this will not affect our access to standard normal medical care.

Name of child.....(add)

Study number: SUK |__|__|__|__|

Project number: BCG |__|__|__|

Name of Parent or Guardian.....
Mother/Father/Guardian

Signature or thumbprint..... Date |__|__|-|__|__|-|2|0|0|__|

I, ...(name),

field worker, declare that I have explained the study to the above participant, that as far as I am aware they have understood well what participation entails and that they freely and willingly give their consent to participate.

Signature..... Date |__|__|-|__|__|-|2|0|0|__|

APPENDIX III: Tuberculosis Information Sheet

Infant BCG study SCC1009

What is TB?

Tuberculosis (TB) is a bacterial disease that most commonly infects the lungs and often causes cough. People who have TB but have not been treated release TB bacteria into the air when they cough. If you breathe in this infected air then you can catch TB.

What are the symptoms of TB?

There are many symptoms of TB and it is often difficult to diagnose. The main symptoms that you should look for are:

Cough > 3 weeks

Fevers

Night sweats / cold clammy perspiration

Anorexia / loss of appetite

Failure to gain weight (children) / progressive weight loss

What to do if you suspect that the child has been exposed to TB?

If you suspect that the child may have been exposed to someone with TB then contact the Sukuta clinic or your field worker as soon as possible so that a home visit can be arranged.

What to do if you suspect that the child has TB?

If your child has any of the above symptoms during the study period then please visit the Sukuta clinic for a medical assessment as soon as possible.

APPENDIX IV: TB Exposure Questionnaire

Infant BCG study SCC1009

TB EXPOSURE QUESTIONNAIRE

Name of child _____

Study no: SUK |__|__|__|__|

Project number: BCG |__|__|__|

Name of interviewee _____

Status (mother / father / guardian / compound head / other)_____

Name of Interviewer_____ Date |__|__|-|__|__|-|2|0|0|__|

In the last 9 months have you, anyone in the household, or any close contacts with the mother / child been:

Diagnosed with TB?	Yes |__| No |__| DK |__|
Taking TB medication?	Yes |__| No |__| DK |__|
Taking a long course of medication (>4 weeks)?	Yes |__| No |__| DK |__|
Had any of the following:	
Cough > 3 weeks	Yes |__| No |__| DK |__|
Fevers	Yes |__| No |__| DK |__|
Night sweats / cold clammy perspiration	Yes |__| No |__| DK |__|
Anorexia / loss of appetite	Yes |__| No |__| DK |__|
Failure to gain weight (children) / progressive weight loss	Yes |__| No |__| DK |__|

If **yes** to any of the above questions then the child has suspected exposure:

Suspected Exposure Yes |__| **No** |__|

Please give details of exposure below and refer child to Dr Adetifa or Dr Flanagan for further action:

_____

_____

Clinical Opinion

Name of clinician _____Date |__|__|-|__|__|-|2|0|0|__|

Comments & Action taken

_____

_____

Continue on study |__| **Remove from study** |__|

If removed from study has the child received BCG? Yes |__| No |__|

If not, give BCG and confirm BCG given

Date BCG given |__|__|-|__|__|-|2|0|0|__|

Given by _____Signature:_____

APPENDIX V: Information Sheet for TST Extension Study

TST extension study L2008.54

Understanding the tuberculin skin test reactivity

We would like to start by thanking you for helping us with the BCG study that you enrolled in previously. We would like to ask your child to have a repeat skin test to compare the results to when the child was younger.

We would like to inform you about why we would like to repeat this test.

What is the Tuberculin Skin Test (TST)? A TST is a test to see if you have had contact with the bacteria that causes tuberculosis (*Mycobacterium tuberculosis, M.tb*). It is a small amount of liquid injected under the skin on the forearm. If your body reacts to the substance in the liquid it will produce a lump on the arm. The size of the lump will be measured by the fieldworkers at 48 - 72 hours after the test. A size greater than 5 mm in average is regarded as positive.

What does a positive test mean? The TST is used to diagnose TB infection but can be positive for the following reasons:

The child is ill with TB disease
The child has been exposed to TB
The child has been vaccinated with BCG
The child has been exposed to bacteria in the environment that look like *M.tb* but are harmless

Why are there so many reasons for a positive TST? The mycobacterium that causes tuberculosis is made up of many small parts, some of these parts are harmless and are contained in lots of other bacteria that are in the environment and are in the BCG vaccine. These harmless parts are contained in the substance that is used in the TST test and that is why your body will react to the skin test when you may not be infected with TB.

What happens if you agree to participate? Your child already had a TST at 4½ months but we would like to repeat the test to see if the results are different when your child is older. We will ask you to come to the Sukuta clinic to receive the test and assess if there is any TB exposure to the child and then come back 48 - 72 hours later to measure the results. Your participation in this study is entirely voluntary.
We will continue to provide vaccines for your child according to the EPI programme and your child will continue to be part of the Sukuta cohort for the following year as stated in the original study SCC1009, regardless of whether you decide to participate in this extension to the study.

What is the aim of the study? To understand how the reactivity to the TST changes over time. We predict that by 20 months the influence of the BCG vaccine on the TST would have reduced and that the test will be more specific to TB exposure or bacteria in the environmental.

Problems. The tuberculin skin test has been widely used for many years and therefore we do not expect any problems with this test. If you have any questions you can come to the MRC clinic at Sukuta at any time to discuss any issue relating to the study with Dr Jane

Adetifa and Ebrima Touray.

Your rights. All information that you give us will be treated as confidential. We will compensate your travel costs to the clinic during this study.

MRC Contacts. If you are unable to attend the clinic on the designated time then please call the Sukuta Health clinic, MRC clinician Dr Jane Adetifa (7700328), fieldworker supervisor Ebrima Touray (7795635), study investigators Sarah Burl (4495442 ext 3021) or Katie Flanagan (4495442 ext 5003). Please feel free to ask any questions you like at any time during the study.

This extension to the SCC1009 BCG study has been approved by the MRC/ Gambian Government Ethics Committees.

If you are willing to help us with this study, please sign the consent form.

Thank you very much.

APPENDIX VI: Consent Form for TST Extension Study

TST extension study L2008.54

CONSENT FORM

The information sheet has been read to me and I understand it / I have read and understood the information sheet.

I understand what participation in the study means for me.

I understand that the information regarding me that is collected in the course of this study will remain confidential.

I understand that if I get sick during the 1 year following recruitment, I can go to the clinic where study staff are providing care, and be examined and treated for free.

I understand that I am free to take part in the study or refuse, and that I can withdraw from the study at any time, and without giving any reason. Deciding not to take part or to withdraw from the study will not affect the care that I am normally entitled to.

I have had a chance to ask questions and have them answered.

Signature or thumb print of parent/guardian: _____

This form has been read by / I have read the above to _____
(write name of parent/guardian)

in a language that he/she understands. I believe that he/she has understood what I explained and that he/she has freely agreed for their child to take part in the study.

Signature of field worker: _____

Name of field worker: _____

Date: |__|__| / |__|__| / |__|__|__|__|

APPENDIX VII: Defining Tregs

Regulatory T cells have received a great deal of attention recently, however the definitive phenotype of Tregs remains controversial, particularly since several markers are also present on activated T cells. The use of FOXP3 expression has improved the classification of Tregs, however not all human $FOXP3^+$ T cells are Tregs. $CD4^+CD25^+$ T cells were shown to be Tregs in mice, but in humans only the $CD4^+CD25^{hi}$ population have regulatory activity (Baecher-Allan, Brown et al. 2003). Unfortunately this population is difficult to gate accurately because there is no clear distinction between $CD25^{int}$ and $CD25^{hi}$ populations, and gating is thus highly subjective. With the advent of FOXP3 as a marker of a Treg cell, a number of different phenotypic subsets have been used in the literature to define Tregs including $FOXP3^+$ T cells as a percentage of all lymphocytes / $CD4^+$ / $CD4^+CD25^+$ or $CD8^+$ T cells; or $CD25^+FOXP3^+$ as a proportion of $CD4^+$ T cells. The further use of $CD127^{low}$ has recently been added to refine Treg definition, but was not used in this thesis.

Our study defined Tregs as the proportion of $CD4^+$ T cells that were $CD25^+$ and $FOXP3^+$. We further analysed for Tregs using a number of different published definitions in order to see whether this would change our results. The variability between Tregs using our definition, and $FOXP3^+$, $CD4^+(FOXP3^+)$ and $CD4^+CD25^+(FOXP3^+)$ Tregs was low (Figure 1A – C). Indeed, the most divergent subset was the $CD4^+CD25^{hi}FOXP3^+$ population which is probably due to the gating difficulties described above. $CD25^{hi}$ T cells also correlated poorly with the other subsets, and showed the greatest within group variation at all time points (Figure 2A – C). This suggests that using the $CD25^{hi}$ definition is likely to give different results to most of the other definitions, and it was not used in this study.

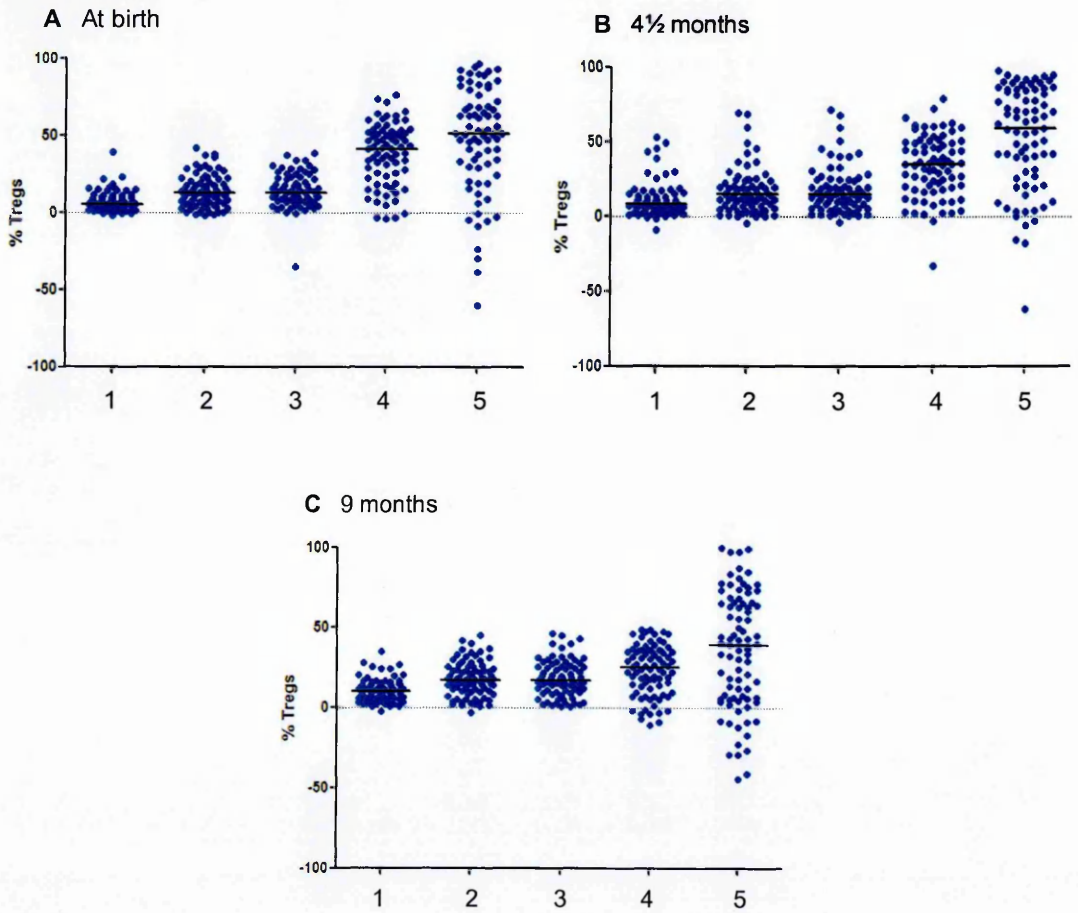


Figure 1: Comparisons between different subsets of Tregs defined by FOXP3 expression at birth (A), 4½- (B) and 9- months of age (C).

1 *%FOXP3 expressing lymphocytes ($FOXP3^+$),*

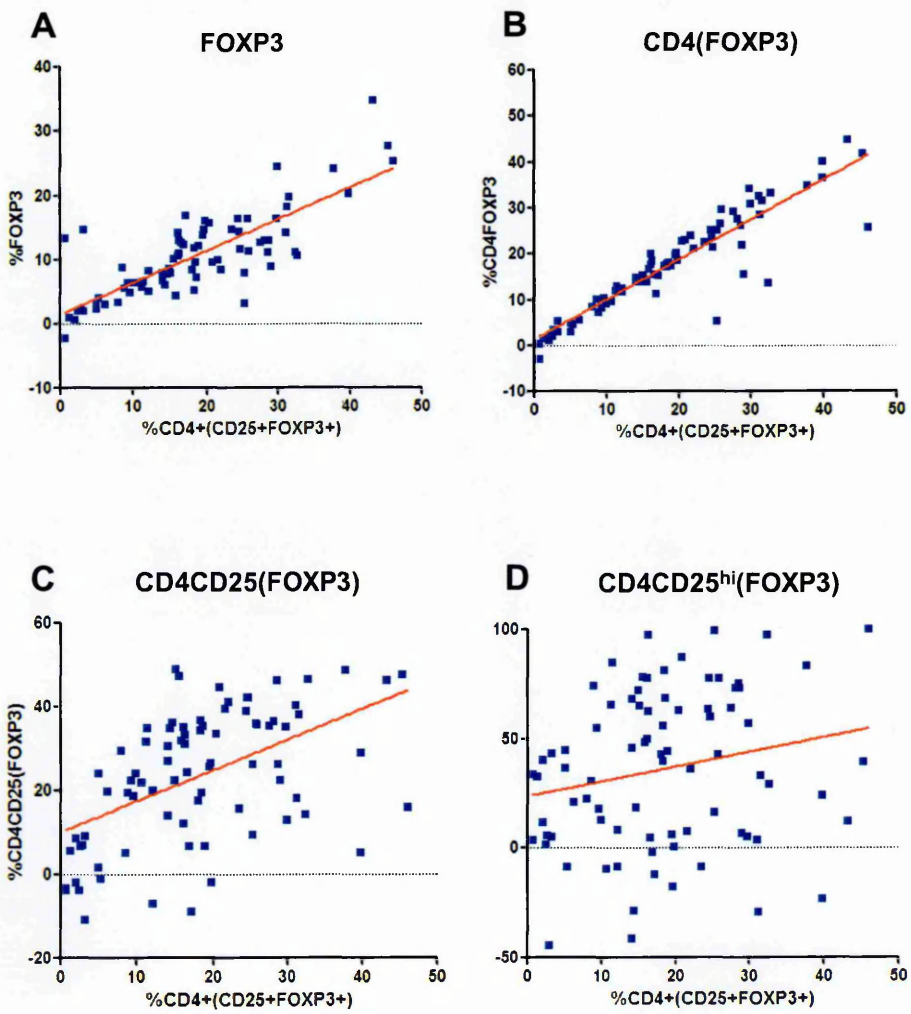
2 *%FOXP3 expressing $CD4^+$ T cells ($CD4^+FOXP3^+$),*

3 *%$CD25^+FOXP3^+$ expressing $CD4^+$ T cells ($CD4^+(CD25^+FOXP3^+)$)*,*

4 *%FOXP3 expressing $CD4^+CD25^+$ T cells ($CD4^+CD25^+(FOXP3^+)$),*

5 *% FOXP3 expressing $CD4^+CD25^{hi}$ T cells ($CD4^+CD25^{hi}(FOXP3)$),*

*population used in our study



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	Lymphocytes ($FOXP3^+$)	CD4 ($FOXP3^+$)	$CD4CD25^+$ ($FOXP3^+$)	CD4CD25hi ($FOXP3^+$)
CD4($FOXP3^+$)	r = 0.8052 p < 0.0001			
$CD4CD25^+$($FOXP3^+$)	r = 0.2889 p = 0.0081	r = 0.6045 p < 0.0001		
CD4CD25hi($FOXP3^+$)	r = 0.2180 p = 0.8449	r = 0.1503 P = 0.1749	r = 0.3447 p = 0.0014	
CD4($CD25FOXP3^+$)	r = 0.7727 p < 0.0001	r = 0.9173 p < 0.0001	r = 0.5206 p < 0.0001	r = 0.2113 p = 0.0599

Figure 2: The relationship between the Treg phenotype used in this thesis ($CD4^+(CD25^+FOXP3^+)$) and other published Treg phenotypes. *Results shown for the 9 month time point in response to SEB stimulation. $FOXP3^+$ (A), $CD4^+(FOXP3^+)$ (B), $CD4^+CD25^+(FOXP3^+)$ (C) and $CD4CD25^{hi}(FOXP3^+)$ (D).Correlations were analysed using Spearmans Correlation coefficient at 5% significance. Table of correlation (r) and p values corresponding to the associations between the different Treg subsets, n = 83*

In conclusion, it is clear that the Treg definition used in our study ($CD4^+(CD25^+FOXP3^+)$) correlates well with those definitions based on FOXP3 expression, but the use of $CD25^{hi}$ as a marker of Tregs is not appropriate due to high inter-individual variability and a lack of correlation between other subsets.